Enabling the dissemination and reuse of proteomics data: status and new projects of the HUPO proteomics standards initiative

Dr. Eric Deutsch

The HUPO Proteomics Standards Initiative (PSI) defines community standards for data representation in proteomics to facilitate data comparison, exchange, and verification. Through yearly workshops and ongoing efforts, we are currently working on updating and releasing new versions of the existing standards mzML, mzTab, mzIdentML, and PSI-MI XML. We have also released new proteogenomics formats proBAM and proBED. Four new standards are under active development. The PSI Enhanced FASTA Format (PEFF) will encode protein sequences along with extensive PTM and variant annotations for the next generation of sequence search engines. A new PSI Spectral Library Format will encode consensus spectra with more metadata that currently possible. A Universal Spectrum Identifier will enable referencing and access to all spectra deposited to any repository. The PROXI API will allow programmatic querying and access to proteomics data across repositories. These past successes and future directions of the PSI will be presented.

Invited Speaker Abstract

Standardising MS Omics for precise and accurate qualifi cation/quantifi cation

Dr. Andrew Percy

LC-MS technology and its associated 'omics methodologies have advanced considerably over the past several years toward improved analyte qualification and quantification. Considering the expanding and continued goals of 'omics researchers, the need for robust tools to assess workflow performance and improve assay development remains essential. This talk aims to provide an overview of new sets of stable isotope-labeled mixtures for metabolomic- or proteomic-centered applications. These collectively afford unique analytical benefits that should help streamline method optimization, system suitability, and quantitative performance in MS 'omics studies. Aspects of their development and application in basic research and clinical translation studies will be presented. Their utility will be highlighted against a few different sample matrices that were prepared and processed using a number of methodologies, LC-MS platforms, and analysis strategies.

Invited Speaker Abstract

Integrated omics for tissue-based mapping of the human proteome

Dr. Cecilia Lindskog

The tissue-based map of the human proteome, generated by the Human Protein Atlas project, focuses on an integrated omics approach for in situ detection of human proteins down to the single cell level. Genome-wide mRNA expression data from three different sources is comprehensively summarized on the interactive webpage and used for categorization of all human genes based on expression level and tissue distribution. The analysis is combined with tissue microarray-based immunohistochemistry, and a large effort is put into extended antibody validation strategies. Recent advances include expansion of the tissue repertoire in the quest for identifying the missing proteins, and in-depth analysis of cell-type specific expression patterns. One such example is expert annotation of all testis elevated genes, to stratify protein expression at various stages of the seminiferous cycle during spermatogenesis.

Invited Speaker Abstract

Omics convergence in cancer research: advances in precision medicine

Dr. Henry Rodriguez

Despite significant progress in understanding cancer through massively parallel sequencing genome programs, the complexity that comprises its diseases remains a daunting barrier. Today we know that molecular drivers of cancer are derived not just from DNA alterations alone, but from protein expression and activity at the cellular pathway level – proteomics. To predict the downstream effects of gene alterations, orthogonal technologies such as next-generation proteomics are needed. This proteogenomics approach (interplay between proteome and genome) is anticipated to transform oncology care from one that relies mainly on trial-and-error treatment strategies based on the anatomy of the tumor, to one that is more precisely based on the tumor's molecular profile. This seminar will discuss how genomics, transcriptomics, and proteomics must all be brought together in the quest to understand the etiology of cancer, in addition to highlighting efforts by the U.S. National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) program in this area of biomedical research. CPTAC's proteogenomics approach was recently successful in demonstrating the scientific benefits of integrating proteomics with genomics to produce a more unified understanding of cancer biology and possibly therapeutic interventions for patients, while creating open community resources that are widely used by the global cancer community. This seminar will also highlight the recently announced APOLLO (Applied Proteogenomics OrganizationaL Learning and Outcomes) program and the efforts of the International Proteogenomic Consortium. APOLLO brings together the U.S. National Cancer Institute, U.S. Department of Defense, and the U.S. Department of Veterans Affairs to create the nation's first healthcare system in which cancer patients will be routinely screened for genomic abnormalities and proteomic information with the goal of matching their tumor type to a specific targeted therapy.

Bioprocess monitoring using quantitative proteomics, glycomics and glycoproteomics

Dr. Jonathan Bones

As routinely used in the field of basic research, quantitative LC-MS based proteomics offers immense potential to better understand biopharmaceutical production using industrial scale mammalian cell culture. A number of examples of the application of quantitative proteomics for bioprocess monitoring will be described. Label free quantitative proteomics was applied to identify markers of cell physiology over the duration of a perfusion bioprocess. Having first identified candidates to monitor apoptosis and necrosis in CHO cells using small scale models, translation into a targeted platform with subsequent verification and validation on the industrial scale were then performed demonstrating superior performance as compared to currently employed assays. Isobaric labelling using tandem mass tags to identify potential markers in response to systematic alterations in bioprocess conditions and associated implications for product quality will also be presented. Finally, the investigations into the CHO cell glycome and its response following exposure to leachable compounds from single use bioreactors will be described.

Invited Speaker Abstract

Psoriatic arthritis through the omics lens

Dr. Vinod Chandran

Psoriatic arthritis is a unique inflammatory arthritis associated with skin psoriasis. In spite of better recognition of this condition and the advent of effective therapy, early diagnosis and disease stratification remain unmet needs in this heterogeneous disease. Genomic studies have led to better understanding of disease pathogenesis. Ongoing transcriptomic, proteomic and metabolomic studies will lead to identifying pathways associated with development of arthritis in patients with psoriasis and likely lead to identifying clinically actionable biomarkers as well as drug targets. My lecture will review the results of omics studies in psoriatic arthritis, the current understanding on disease pathogenesis, the current status of biomarker discovery, challenges faced, promising markers identified, and the way forward.

Tissue and plasma proteomics allows early stage colorectal cancer detection/stratifi cation that improves patient outcome

Prof. Mark Baker

Mark Baker: Born 1956 Sydney and completed PhD 1985 at Macquarie University. Main research focus is biochemistry of proteins in human health/disease. Mark made stellar contributions to the growth of proteomics societies nationally, regionally and internationally from the outset, including a successful stint in the USA biotech industry. In 2004, he co-founded the Australasian Proteomics Society, was a founding AO-HUPO Councillor and foundation member of HUPO. Mark returned from the USA to grow Australia's national proteome facility APAF as its CEO, securing major government support for systems biology infrastructure. Mark serves on the HUPO Board from 2005 until now. He was elected to the HUPO Executive in 2012 and served as the i8memdiate past HUPO President. in 2013. Mark co-chaired the 9th HUPO World Congress in Sydney when the Human Proteome Project was launched. He has been a chair/member of many HUPO initiatives and management committees, as well as sitting on prestigious journals' Editorial Boards and industry Advisory Boards. Mark was recognised in 2012 with the HUPO Distinguished Service Award. He has published ~150 peer-reviewed papers, graduated more than 38Honours/PhD graduates and is the inventor of 5 technology patents. He remains an advocate for industry:academic collaborations, media engagement and promotion of young researcher career paths. He is passionate about cooking, epicure, fishing, Rugby and biomedical research.

TMT-based proteomic analysis identifi es novel viral evasion pathways

Prof. Paul Lehner

Evolutionary pressure has produced an “arms race” between cellular restriction factors that limit viral replication and viral proteins which overcome host restriction. Thus viruses need to manipulate host cell signaling pathways to enable their replication and evade immune recognition. In turn, infected cells must sense and respond appropriately to intracellular viral infections. Many virus-induced changes in the host proteome are post-translational and therefore lend themselves to proteomic analysis. We have adapted functional proteomic approaches to identify receptors manipulated by viruses and identify key components of intracellular signaling pathways. We applied ‘Plasma Membrane Profiling’ to gain a systematic unbiased overview of cell surface receptors manipulated by viruses, as well as TMT-based proteomic analysis to delineate virus-induced changes in the total cell proteome. TMT-based quantitation allows us to create a dynamic temporal map of receptors manipulated by both integrating (HIV) and non-integrating (herpes) viruses. This approach has established novel paradigms of viral interference and manipulation of the host immune system which will be discussed.

Invited Speaker Abstract

Quantitative proteomics in a wellness and disease setting

Prof. Robert Moritz

The convergence of advances in systems medicine, big data analysis, individual measurement devices, and consumer-activated social networks has led to a vision of healthcare that is predictive, preventive, personalized, and participatory (P4), also known as 'precision medicine'. In order to understand the basis of wellness and disease, we have pursued a global and holistic approach termed 'systems medicine'. The defining feature of systems medicine is the collection of diverse longitudinal data for each individual. These data sets can be used to unravel the complexity of human biology and disease by assessing both genetic and environmental determinants of health and their interactions. We refer to such data as personal, dense, dynamic data clouds: personal, because each data cloud is unique to an individual; dense, because of the high number of measurements; and dynamic, because we monitor longitudinally. The genome provides the basis of which predictions of wellness can be made and according to population statistics, propensities of disease can be assumed. Of all the measurements, proteins are indicators of the current health status and their reliable, comprehensive and quantitative measurement is key to unlocking the trajectories of wellness. To perform large scale quantitative proteomic experiments over hundreds of samples with robust operation, reproducibility across different complex matrices, a system that is well characterized both in terms of data collection and data analysis is paramount. I will discuss our methods in attaining high accuracy and signal stability in label free proteomics enabling the processing of many hundreds of samples in an automated unattended operation. Our latest methods in SWATH-MS operation and data analysis with superior higher performance characteristics and steps to achieve these types of results and operational specifics for wellness and disease studies will be discussed.

Invited Speaker Abstract

Instrumentation and methods for the identifi cation and sequence analysis of intact

Prof. Don Hunt

This lecture will focus on data generated with an ion source that facilitates simultaneous generation of positively charged sample ions by electrospray ionization and negatively charged reagent ions for both electron transfer dissociation (ETD) and ion-ion proton transfer (IIPT) reactions on Orbitrap mass spectrometers. Implementation of multiple C-trap fills for enhanced sensitivity will be discussed and both parallel peak parking, and ion ejection strategies to facilitate protein separation and enhanced sequence coverage of intact proteins will be described. Use of IIPT/ETD facilitates near complete sequence coverage on many intact proteins, including antibodies, and is ideally suited for locating multiple posttranslational modifications on the same protein molecule. Also discussed is the identification of posttranslationally modified, Class I MHC peptides that trigger central memory T-cells to kill cancer cells.

Quantitative interaction proteomics: Insights into biological systems

Dr. Ben Collins

Protein complexes and protein interaction networks are essential mediators of most biological functions. Complexes supporting transient functions such as signal transduction processes are frequently subject to dynamic remodelling. Currently, the majority of studies on the composition of protein complexes are carried out by AP-MS and present a static view of the system. We have previously used AP with SWATH-MS to interrogate signaling complexes in perturbed systems (Collins et al. Nature Methods 2013). I will describe new applications of this strategy in relevant biological systems. Firstly I will show how quantitative analysis of a disease related mutant of the ubiquitin-directed AAA-ATPase p97 has led to the discovery of new substrates in addition to insights into the modularity of p97 adaptor proteins. Second, I will show that, combined with transgenic mouse technology, AP-SWATH allows to examine dynamic re-organization of signaling complexes in developing or mature primary T cells after stimulation.

Invited Speaker Abstract

Pulse Azidohomoalanine (AHA) labeling in mammals (PALM) analysis for global

Prof. John Yates

Identifying changes in animal models of disease at the earliest time point prior to pathology is desired because these alterations are more likely to cause the pathology. At these early time points alterations in protein expression may be difficult to identify hidden by the overwhelming static proteome. One possible solution is to identify only newly synthesized proteins (NSP) within a discrete time period. We developed PALM analysis to identify and quantify NSP from rodent tissues by mass spectrometry. When introduced by Tirrell and Schumann it was also referred to as BONCAT. A rodent diet was developed where methionine was replaced with AHA. Mice were given the PALM diet for different time periods and multiple tissues were tested for incorporation of AHA. To quantify the NSP, a heavy biotin-alkyne and heavy AHA were synthesized. The use of these reagents will be discussed in animal models and cell culture experiments.

Viral infection-driven dynamics of proteome organization

Dr. Ileana Cristea

The coexistence and coevolution of hosts with pathogens is intrinsic to our ecosystem. Pathogenic infections induce an array of changes in the hosts that are tightly linked to the progression of infection and establishment of disease. At the cellular level, this is reflected in alterations in host cell composition, organization, and ability to communicate with other cells. Thus, changes in the host proteome, metabolome, lipidome, and secretome have started to be recognized as signatures of infectious or disease states. These alterations function to either induce host defenses that counteract the infection or promote pathogen replication for spread of infection. Consequently, the discovery and characterization of these signature changes are essential for both understanding the biology of infection and identifying novel targets for therapeutic interventions. This presentation will highlight the value of advanced mass spectrometry-based proteomics for defining the dynamics of proteome organization and understanding cellular defense mechanisms during viral infections.

Invited Speaker Abstract

Proteogenomic insights for cancer biology, prognosis and treatment

Dr. Karin Rodland

The flow of information from the genome to the phenome is not linear, but intricately regulated at the level of transcription, translation, post-translational modification and cellular localization. A full understanding of the biological changes driving cancer initiation, progression, and response to therapy requires comprehensive analysis at all levels of information, and meaningful integration into pathways and networks. The Clinical Proteomic Tumor Analysis Consortium has provided a mechanism for adding comprehensive data on protein abundance and post-translational modifications, closely linked to comprehensive genomic and transcriptomic data, in previously analyzed TCGA tumor samples and purpose-collected prospective samples. This talk will present novel insights from the proteogenomic interrogation of high grade serous ovarian carcinoma, including pathways associated with clinical outcome, and describe how the same approaches can be applied to patient samples from on-going clinical trials to provide dynamic protein-level information on the development of drug resistance.

Invited Speaker Abstract

Proteomics, embracing the chaos

Prof. Juri Rappsilber

As a PhD student I learned the three most important indications for the function of an uncharacterised protein: location, location, location. Or is it? As analysis depth increases so does the number of out-of-context proteins identified at any location in the cell investigated. Of course, analysis by proteomics requires prior isolation of a cellular structure/organelle. Are cells tidy and experiments dirty? Here we analyse chromatin by proteomics and challenge the black/white logic of proteomic lists and GO annotations. Interestingly, this departure shows a more direct root to protein function and may affect our view of the cellular interior.

Invited Speaker Abstract

The surfaceome of human cells: a sweet source of novel immunophentotyping and immunotherapy targets

Dr. Rebekah Gundry

The surfaceome is a rich source of accessible live cell markers for targeted drug delivery, immunotherapy, and cell sorting without requiring genetic modifications. Despite its utility and critical roles in biology and disease, the surfaceome has not yet been defined for most human cell types. Using a selective chemoproteomic approach termed Cell Surface Capture Technology, we are able to generate a unique view of the cell surface N-glycoproteome of any mammalian cell type. Combining surfaceome data from disparate cell types within the Cell Surface Protein Atlas, new targets for immunophenotyping and immunotherapy are rapidly identified. To date, we have applied this strategy to identify new cell type specific markers for stem cell derivatives and primary cells of multiple developmental stages, lineages, and diseases – from cardiac fibroblasts, cardiomyocytes, epithelial cells, and hepatocytes to blood cancers. In progress are new approaches to facilitate the systematic mapping of surfaceomes across a broad range of cell types, including those in limited supply, to enable quantitative studies of surfaceome dynamics during development and disease.

Serum N-glycome – a biomarker for ovarian cancer diagnosis

Prof. Veronique Blanchard

Ovarian cancer (OC), the sixth most common cause of cancer deaths in women, is frequently diagnosed at a late-stage. Current diagnostic methods of OC show only a moderate sensitivity especially at an early-stage of the disease. Therefore, better biomarkers are needed to improve the diagnosis of OC.
In this work, N-glycans were released from serum proteins, permethylated and measured by MALDI-TOF-MS. Serum glycome modifications observed in OC patients by MALDI-TOF-MS could be combined as a glycan score named GLYCOV that was calculated from the relative areas of the 11 N-glycan structures that were significantly modulated. GLYCOV showed an improved sensitivity and specificity at any stage of the disease and an improved discrimination between malignant and benign ovarian tumors, which is of high relevance to clinicians as it is challenging to diagnose malignancy prior to operation (1,2). The glycome of ascitic fluid also showed typical features of inflammatory conditions (3).

Immunoproteomic approaches to host-pathogen interactions

Prof. Dave Goodlett

The innate immune homeostasis, involving a balance between an effective host defense response and associated inflammation, is a complex, dynamic process that facilitates the very first line of defense against invading pathogens. Predicting how immune phenotypes are altered and understanding a global immune landscape is to date difficult. Thus, we have set out to establish a comprehensive, quantitative evaluation of the whole system to help predict phenotypes that arise when the mammalian immune system confronts an intruder. Here we use Burkholderia thailandensis as a model to understand proteome reorganization during biofilm formation and how that may help bacteria influence antibiotic sensitivities. Using a combination of cytokine profiling, global (phospho) proteomic analyses, and network biology approaches, we decipher how macrophages exhibit distinct, disparate phenotypes in response to endotoxin challenges.

Proteomics for study of liver diseases

Prof. Barbara Sitek

Intrahepatic cholangiocarcinoma (ICC) and pancreatic ductal adenocarcinoma (PDAC) are highly aggressive cancer types that arise from epithelial cells of the pancreatobiliary system. Owing to their histological similarity, differential diagnosis between ICC and metastases of PDAC located in the liver (mPDAC) frequently proves an unsolvable issue for pathologists. Yet, differential diagnosis of ICC and mPDAC is highly relevant as it leads the patient either to possibly curative resection (for ICC) or to palliation (for mPDAC). Therefore, in this study we were focused in identification of new biomarkers for differential diagnosis. Moreover we identified MFAP4 as a serum biomarker candidate for hepatic fibrosis and cirrhosis in hepatitis C patients. The aim of the present study was to elucidate the potential of MFAP4 as biomarker for hepatic fibrosis with a focus on the differentiation of no to moderate and severe fibrosis stages and cirrhosis.

The proteomics of protein kinase signaling

Dr. Michael Yaffe

In this talk I will summarize our progress elucidating the phosphorylation motifs for the entire human kinome using oriented peptide library screening approaches and computational analysis of mass spectrometry datasets. I will showcase the current version of Scansite (https://scansite4.mit.edu/4.0/#home), a free web-based bioinformatics tool that links protein phosphorylation sites to their likely upstream kinases, allowing construction of signaling pathways in silico. Finally, I will present new data highlighting motifs and substrates of poorly understood families of mitotic kinases, and show unexpected roles for the DNA damage-responsive kinases Chk1, Chk2, and MK2 in a cytoskeletal-mediated adaptive response to genotoxic stress.

Subcellular proteomics and friends; fi nding lipid raft-dependent RNA-binding proteins that regulate exosome microRNA cargo selection

Prof. Michelle Hill

Extracellular vesicles (EV) including exosomes and microvesicles mediate intercellular communication by delivering functional biomolecules including microRNA (miRNA) to recipient cells. We previously linked the putative tumour suppressor action of cavin-1/PTRF in prostate cancer with altered EV miRNA and lipid raft membrane micodomain proteome. Cavin-1 selectively reduced the EV levels of a subset of miRNAs, independent of their cellular levels. We hypothesized that selective miRNA loading is mediated by cavin-1 induced lipid raft RNA-binding proteome changes. To test this hypothesis and identify the specific RNA-binding protein, we applied subcellular proteomics, EV small RNA sequencing, computational and multiple cell biology techniques to identify a cavin-1 regulated RNA binding protein and a novel RNA motif that could mediate the cargo loading. Overall, this presentation will demonstrate an integrative subcellular systems biology approach to address key molecular mechanisms

Invited Speaker Abstract

Using proteomics to explore the pro-invasive crosstalk between stromal and cancer cells

Dr. Sara Zanivan

The tumour stroma is composed of many different cell types that work in concert to generate a microenvironment permissive to tumour initiation, progression and formation of metastasis. Cancer associated fibroblasts (CAFs) are amongst the most abundant non-neoplastic cells in solid tumours and are unique for their capability to secrete a plethora of factors that alter the behaviour of cancer and stromal cells. Thus, CAFs play a key role in cancer pathology. We use quantitative MS-based proteomics to map the secretome of CAFs and identify the molecular mechanisms that underpin its pro-invasive functions. We have identified unprecedented mechanisms through which CAFs dictate tumour invasion. I will discuss some recently published and current efforts in understanding how to target CAF in cancer.

Invited Speaker Abstract

Perspectives of proteomics in laboratory medicine and total lab automation

Prof. Andrea Urbani

Laboratory medicine is swiftly moving forward in the field of total lab automation associating hub centers, main operations able of running millions of test per years, with spoke structures distributed on the territory. In the last five years a number of proteomics initiatives have been opening new routes in the development of new clinical deliverables. These associate the key concepts of Proteomics which links together two fundamental ideas: the thorough investigation of protein structures with a multifactorial integration of data. Nevertheless the sustainability of these diagnostic products in the current healthcare systems and the current driving force toward more efficient clinical laboratory settings are often not fully discussed. This presentation will touch and possibly integrate these two worlds providing some examples from one of the largest clinical chemistry operation facility in Italy, the Gemelli Hospital clinical laboratory, running about 6.5 Millions tests per year. The potentiality of some Proteomics deliverables will be introduced and discussed in the light of the current analytical performances of different technological platforms and cost effectiveness.

Clinical proteomics: my adventures in wonderland

Prof. Daniel Chan

During the last decade of proteomic research, significant progress has been made in the advancement of new technologies and the discovery of potential biomarkers. However, limited successes have been shown in the translation of proteomic discoveries into clinical practice. Clinically, one of the major goal of cancer biomarker discovery is to detect early stage lethal cancer. In my presentation, I will take you with me through the journey of my personal adventures in the wonderland of clinical proteomics. I will discuss our strategies and give specific examples for biomarker discovery, validation to translation and our research partnerships with industry, the National Cancer Institute (NCI) Early Detection Research Network (EDRN) and the Clinical Proteomics Tumor Analysis Consortium (CPTAC) in linking genomics with proteomics. The successful translation of clinical proteomics into clinical practice will require close collaboration between researcher, industry, regulator and clinician/clinical laboratory.

Invited Speaker Abstract

Remodelling of proteome expression and turnover during cell transformation

Prof. Angus Lamond

We have used a 'Multidimensional Proteomics' approach (see Larance and Lamond, Nat Rev. Mol. Cell Biol. 2015) to conduct an in-depth, quantitative analysis of the change in the proteomics landscape of healthy human epithelial cells that are induced to transform in culture by activation of the tyrosine kinase activity of the v-Src oncoprotein. We have characterized the proteome of untransformed MCF10A cells to a depth of ~14,000 proteins, detecting over 350,000 separate peptides with a mean coverage of >25 peptides per protein. These cells were induced to transform in culture by activation of v-Src, resulting in major phenotypic changes within 48-72 hours. The transformed cells show many of the hallmark phenotypes of metastatic cancer cells seen in the clinic, including changes in morphology, loss of contact inhibition and increases in both migration and invasiveness. We have quantitated by label free MS analysis the response kinetics for the entire MCF10A proteome, measuring seven time points, in biological triplicate, spanning 1-72 hours post v-Src activation. We have also used pulsed SILAC to measure, in biological triplicate, the rates of protein synthesis, degradation and turnover in both healthy and v-Src transformed MCF10A human epithelial cells. All of these data have been integrated in a searchable, online database featuring user-friendly, interactive data exploration tools (see www.peptracker.com/epd). I will present an overview of the resulting data, which provide a detailed insight at a systems level into the molecular events and remodeling of metabolism and gene expression that accompany the formation of a cancer cell. I will also relate these data from a human cellular model to clinical data and healthcare records.

Integrated analyses of epigenetic complexes and networks

Dr. Michael Washburn

Chromatin remodeling proteins, complexes, and networks paly critical roles in human biology and diseases. We integrate multiple technologies to study these systems. To begin, proteins of interest are affinity purified using the multi-functional HaloTag, which allows us to make one cell type and conduct multiple experiments. Using MudPIT and the dNSAF label free quantitative proteomics approach, we determine protein complex content and protein interaction networks, which we visualize using advanced computational tools like topological data analysis. We then employ state of the art microscopy techniques to analyze proteins of interest to analyze interactions in live cells. To gain deeper insight into these complexes and networks we will often perturb the systems using gene deletions, truncations, mutations, or drug treatments, for example. A major focus of the group is currently on the human Sin3 histone deacetylase (HDAC) containing complexes and their perturbation by the anticancer drug Vorinostat.

Bridging the gap in oncology diagnostics: converting omic data into clinically relevant assays

Prof. William Gallagher

Bridging the Gap in Oncology Diagnostics: Converting Omic Data into Clinically Relevant Assays
The effective implementation of therapeutic regimens in the oncology arena depends on the successful identification and translation of informative biomarkers to aid clinical decision-making. Antibody-based proteomics occupies a pivotal space in the cancer biomarker discovery and validation pipeline, facilitating the high-throughput evaluation of candidate markers1. Indeed, it is vital that if antibodies are to be utilised for clinically assays, especially immunohistochemistry, that appropriate validation procedures are employed to ensure specificity2. In addition, reverse engineering of transcriptional networks using gene expression data enables identification of genes that underpin development and progression of different cancers3. Such approaches also provide a potential means to elucidate robust biomarkers. Here, several case studies will be provided in respect to efforts made to transition omic-based discovery data towards clinical utility, particularly in the context of oncology diagnostics.

Targeted glycoproteomics for novel bladder cancer biomarkers: a step towards precision oncology

Dr. José Alexandre Ferreira

Bladder carcinogenesis and progression is accompanied by profound alterations in protein glycosylation at the cell surface, holding potential to improve disease management. Searching for prognosis biomarkers and novel therapeutic targets we have disclosed that advanced bladder tumours and corresponding metastasis express the sialyl-Tn (STn) antigen, which stems from a premature stop in O-glycosylation. STn overexpression was triggered by hypoxia and modulated protein functions in ways that favored motility and invasion, while promoting immune escape. A glycoproteomics approach based on Vicia villosa lectin-affinity chromatography enrichment and nanoLC-ESI-MS/MS supported by in silico data curation (NetOGlyc, Phanter, Oncomine, Cytoscape) identified several key cancer-associated glycoproteins (MUC16, CD44, integrins) carrying this posttranslational modification. In particular, MUC16 STn+-glycoforms were found for the first time in a subset of advanced-stage bladder tumours facing the worst prognosis. These observations support the existence of unique bladder cancer glycosignatures that should be comprehensively explored envisaging molecular-based precision oncology.

Invited Speaker Abstract

Chemical proteomics reveals the target space of clinical kinase inhibitors

Prof. Bernhard Kuster

Kinase inhibitors have developed into important cancer drugs because de-regulated protein kinases are often driving the disease. Close to 40 such molecules have been approved for use in humans and several hundred are undergoing clinical trials. As most compounds target the ATP binding pocket, drug selectivity among the 500 human kinases is a recurring question. Clinicallyspeaking, polypharmacology can be beneficial as well as detrimental. Therefore, knowing the full target spectrum of a drug is important but rarely available. We have used a quantitative chemical proteomics approach to profile 240 clinical kinase inhibitors in a dose dependent fashion in cancer cell lysates to identify thousands of drug-protein interactions. This presentation will outline how this information is generated and how it can be used to identify molecular targets of toxicity, re-purposing existing drugs for new indications, finding combinations of drugs to overcome resistance or provide starting points for new drug discovery.

Knowledge mining from -omics datasets in the era of big data

Prof. Fuchu He

Throughout the history of natural science, it is definite that the discovery of our knowledge and disciplines are triggered by the unprecedented scale and speed of big data and achieved by efficient mathematical strategies.
In the past 25 years, mathematical strategies have been used in my laboratory to generate multiple biological findings based on large-scale datasets. The story began with simple statistical methods used to find four periodic phenomena of molecular evolution in the early years.
Next, machine learning strategies, such as naïve Bayesian network, have been used to find the instinct features of proteome organization, especially the protein interactions.
At the current stage, clustering strategies are playing important roles for the molecular characteristics of HCC and new personalized treatment strategies based on large scale human proteome datasets.
The era of big data will bring in new insights in life sciences and present new opportunities in research. Artificial intelligence strategy will play dominant roles in the coming knowledge discovery. My team is now engaged in developing an automatic knowledge discovery highway for grand knowledge.

Invited Speaker Abstract

False discovery rates in proteomics: a tale of two extremes

Prof. Lennart Martens

A lot of attention in proteomics (as in any high-throughput analytical field) is spent on controlling the false discovery rate (FDR). Over the past several years, we have investigated in detail many of the issues faced in the more extreme proteomics studies (e.g., multi-organism proteomics, metaproteomics, proteogenomics), and in doing so have discovered both the strengths and the limitations of current approaches. Here, two novel approaches will be presented that each tackle a different kind of problem in accurate FDR control: an approach to control the FDR for small sub-samples, as for instance encountered in host-pathogen studies, and the first ever approach that can verifiably control FDR accurately in open-ended modification searches while maintaining high sensitivity and high speed.

Invited Speaker Abstract

We are not alone: the roles of farm animal proteomes for human health

Prof. Emoke Bendixen

The OneHealth perspective reminds us that human life and health depends on the health and biology of countless animals, plants and microbes. This has become particularly urgent and clear with the rapid spread of microbial resistance to antibiotics (AMR), which currently poses a major threat to global human health. AMR is a direct consequence of a massive use of antibiotics both for humans and farm animals. With around 70 % of the total global antibiotics consumption being used for farm animals, solving the antibiotics crisis will depend on solving the current crisis in farm animal health. Moreover, 60 % of all human pathogens originate from animals., and while some instances are highly publicized such as SARS and H5N1, the area of zoonosis is largely neglected. Comparative studies between humans and animals will be essential to deliver the best possible measures to control infectious diseases. Research that leads to fundamental understandings of processes such as host response to pathogens in farm animals is of key importance. Funders and scientists must prioritize farm animal health research at the same level as human health research, to protect future antibiotic resources. Today this is very far from reality.
Meeting the need for new antibiotics and reducing the use of current antibiotics requires new lines of research, including fundamental research into zoonosis, studies of pathogen biology and comparative infection studies of humans and animals. For this, proteome research of farm animal species is clearly needed, in part to study specific host-pathogen crosstalk at the molecular level, but also for making available panels of accurate health measures for monitoring the health state of relevant animal models and for delivering proof of concept when new drugs, or alternatives like pre- and pro-biotics are being tested for their efficiency in protecting both animals and humans against pathogens. This talk will present our long time studies of host pathogen interactions in cows and pigs, including studies of milk pathogens in cows, and gut pathogens in pigs. One example is a recent study of how porcine FUT1 gene variants affect pigs resistance to E. coli F18 infections, by influencing health, growth, microbiome and glycan structures in the pig gut. Such pig models also provides the opportunity to study comparative host pathogen interactions in pigs and humans, and to study e.g. virulence factors of E. coli.

Dissecting phosphotyrosine signaling networks by quantitative phosphoproteomics

Prof. Jesper Olsen

Quantitative phosphoproteomics is emerging a powerful technology for global analysis of cellular signaling networks. In particular tyrosine phosphorylation (pTyr) is of great importance in eukaryotic cells due to its crucial role in regulating intracellular signaling networks controlling cell fate decisions such as proliferation, migration, differentiation, cell cycle progression and apoptosis. Here we employed quantitative phosphoproteomics to delineate receptor tyrosine kinase (RTK) signaling dynamics activated by different ligands leading to differential cellular outcome. We quantified thousands of pTyr events as a function of ligand and stimulation time and revealed RTK-specific regulation of pTyr sites on key adaptor and signaling molecules, which fine-tune cell migration and proliferation. These results, based on a multidisciplinary approach, which combines quantitative phosphoproteomics and functional assays, identify ligand-dependent mechanisms for the control of RTK signaling and for the specification of long-term cellular outcomes.

Towards a blood-based diagnostic biomarker panel for bipolar disorder

Prof. Sabine Bahn

The diagnosis of Bipolar Disorder (BD) and Major Depression (MDD) is based on the subjective reporting of symptoms.
Around 60% of BD patients are misdiagnosed and the average time until a correct diagnosis is achieved is 9.6 years. Objective blood-based biomarkers could help in the differential diagnosis of BD.

We have previously identified a panel of 20 protein biomarkers in serum capable of differentiating BD from MDD patients using a multiplex ELISA platform. However, multiplexed ELISA is costly and antibody batch variation is associated with problems in reproducibility.
Thus, we are now developing a two-stage diagnostic approach combining a highly scalable digital clinical app, which assesses symptoms and demographic patient characteristics, in conjunction with a biomarker test using multiplex MRM mass spectrometry on self-collected dried blood spots (DBS).

If our results can be validated, this novel diagnostic approach would represent a cost-effective solution with the potential to dramatically reduce misdiagnosis rates and improve patient outcomes.

Remodeling of the SUMO proteome in response to proteotoxic stress

Prof. Ron Hay

Centre for Gene Regulation and Expression, University of Dundee, UK
Many cancer treatments rely on the induction of proteotoxic stress for therapeutic activity. Arsenic trioxide, bortezomib (proteasome inhibitor) and heat stress are all used to treat different cancers and are also know to trigger changes in modification of proteins by the small ubiquitin-like modifiers (SUMOs). We developed a specific peptide enrichment strategy that enables the identification of SUMO acceptor lysine residues on a proteome-wide scale. Conjugation to substrates of a SUMO-2 variant with Threonine 90 mutated to Lysine produces a Gly-Gly remnant on target lysines after cleavage with endoproteinase Lys-C. This allows specific enrichment of SUMO-modified peptides using diglycyl-lysine antibodies. Furthermore, it leaves a diagnostic signature that cannot be confused with other modifications. This has allowed the direct identification of over 6000 target lysines in 2000 human proteins in response to heat stress. These data help us to build models to explain the substrate specificity of SUMO modification

Drug resistance assessed by mass spectrometry based omics technologies

Dr. Simone Lemeer

Targeted therapies against oncogenic receptor tyrosine kinases are showing promising results in the clinic. However, despite the initial response, most patients become resistant. How are cells able to survive initial treatment? A multi-omics approach was used, including mass spectrometry based proteomics, phosphoproteomics, kinomics and metabolomics, to gain more insight in regulated processes during early TKI treatment. Our findings demonstrate how this multi-omics approach contributes to a better understanding of the molecular pathways underlying immediate drug tolerance and elucidates new potential targets that can be co-inhibited to prevent resistance development. In addition to this, I will present a universal and optimized phosphoproteomic workflow that enables comprehensive analysis of signaling pathways, yielding 60% gain in identifications without additional analysis time compared to previous workflows.

Proteome landscape of hepatocellular carcinoma

Prof. Ying Jiang

Hepatocellular carcinoma (HCC) is a highly lethal cancer, ranking as the fifth most prevalent tumor and the second leading cause of cancer-related deaths worldwide. In China, chronic infection with hepatitis B virus (HBV) is one of its major risk factors, most patients are diagnosed at advanced stages of HCC with poor survival. A comprehensive molecular view of cancer is necessary for understanding the underlying mechanisms of disease, improving prognosis, and ultimately guiding treatment. Here we performed a comprehensive mass-spectrometry-based proteomic characterization of HCC tumors and paired no tumor tissues. Based on protein profiling data, those HCC cases were stratified into three subtypes. Proteomic alterations in signature molecules/pathways and networks among subtypes were analyzed to decipher the molecular characteristics of HCC and contribute to new personalized treatment strategies.

Invited Speaker Abstract

Improved sensitivity and specifi city of proximity-dependent biotinylation approaches

Dr. Akhilesh Pandey

Proteins rarely function in isolation as they often occur as components of multiprotein complexes. Mass spectrometry-based proteomic approaches offer an opportunity to identify protein-protein interactions in an in vivo setting in a sensitive fashion. However, a major limitation of immunoprecipitation-based strategies for identifying protein interactors is that it is difficult to detect transient interactions as well as interactors that are present in low abundance in cells. To circumvent this, proximity-dependent biotinylation strategies (e.g. BioID) are being increasingly used where the bait protein is fused to a promiscouous biotin ligase, BirA, leading to biotinylation of the interacting proteins. In this approach, the biotinylated proteins are generally identified by digesting proteins captured by streptavidin beads which leads to identification of proteins although biotinylated peptides are not directly identified. We have recently developed a method for direct capture of biotinylated peptides which increases both the sensitivity and specificity of experiments that employ this biotinylation-based approach. I will discuss our recent studies using this strategy for interactome analysis using isogenic systems for analysis of signaling pathways.

The human protein atlas - implications for human biology, drug development and precision medicine

Prof. Mathias Uhlen

The human proteins constitute the major building blocks for the function of the various processes necessary for human life. We have classified all the protein coding genes in humans using a combination of genomics, transcriptomics, proteomics and antibody-based profiling. We have classified all the protein coding genes in humans using a combination of genomics, transcriptomics, proteomics and antibody-based profiling and used this data to study the global protein expression patterns in human cells, tissues and organs. A Tissue Atlas was launch in 2014 (Uhlen et al, Science, 2015), a Cell Atlas in 2016 (Thul et al, Science, in press) and a Pathology Atlas will be launched in 2017. Recently, we have also set-up an animal cell factory using CHO cells using synthetic biology and high-throughput expression systems with the aim to produce full-length proteins representing all the 2,900 secreted proteins encoded in human genome.

Invited Speaker Abstract

Proteogenomic analysis of diff use gastric cancers

Prof. Daehee Hwang

We report an integrated proteogenomic analysis of diffuse gastric cancers. Genomic alterations supported by proteomic data enabled prioritization of cancer genes associated with patient survival. Correlations between mRNA and protein abundance changes also enabled selection of oncogenes and tumor suppressors affecting patient survival. Integrated analysis of mRNA, protein, phosphorylation, and glycosylation data identified four subtypes of gastric cancers with subtypes 1-4 associated with cell proliferation, immune response, metabolism, and invasion, respectively; subtypes 1/3 and subtypes 2/4 distinguishable only by proteomic data; and association of subtypes 2 and 4 with immune- and invasion-related pathways identified uniquely by N-glycoproteome and/or phosphoproteome data. Finally, correlation between mutation and phosphorylation enabled effective identification of mutation-signaling interplays associated with subtype-dependent patient survival. Therefore, integrated proteogenomic analysis affords more enhanced understanding of cancer biology and patient stratification than genomic analysis alone.

Probing biopharmaceutical proteins and protein assemblies by hybrid mass spectrometry approaches

Prof. Albert Heck

Mass Spectrometry based proteomics has played a pivotal role in revealing the plethora of protein interactions and post-translational modifications that take place inside a cell, wherein proteins form protein assemblies and/or signalling networks. Especially using affinity purification of a tagged proteins followed by mass spectrometric analysis of its binding partners a wealth of data has been gathered revealing the all-embracing protein networks present in cells. Following the charting of all these interactions, a next step will be to now gather more in-depth structural and functional information on these individual protein assemblies. This may come from in-depth high-resolution structural models, as well as detailed information on how they function and dynamically evolve during cellular perturbations. Mass spectrometry may also contribute to this next level of protein interaction analysis although it does require partly different and novel approaches. To contribute to this emerging new area in proteomics, our group is developing new methods using native mass spectrometry and cross-linking mass spectrometry with the aim to bridge the gap between interaction proteomics and structural biology. These new innovations and applications of them in interaction proteomics will be central in this presentation.

In the first part of the talk native mass spectrometry and its applications in probing protein assemblies and interactions will be described, focusing on examples wherein the dynamic assembly of a protein complex involved in the circadian timing in cyanobacteria will be highlighted. Herein, by using a combination of native, HD exchange and cross-linking mass spectrometry and cryoEM, we were able to define a novel structural model improving our understanding of the circadian rhythm. Additionally, novel developments in MS instrumentation for native MS will be highlighted, especially a new Orbitrap based instrument that offers high-sensitivity and mass resolution, allowing an in-depth detailed analysis of glycoproteins, viruses and even whole intact ribosomes.

The second part of the talk will highlight our recent work on cross-linking mass spectrometry. Cross-linking combined with mass spectrometry (XL-MS) provides another powerful approach to probe the structure and interaction profile of protein assemblies. Up to now XL-MS has been primarily limited to the characterization of purified protein assemblies. We have set out to develop XL-MS methods aimed at probing protein interactions at the proteome level, using complete cell lysates or whole organelles as starting material. We, therefore, combined several novel innovative methods to address some of the hurdles in this field. These innovation include the use of a low energy CID cleavable cross-linker, novel hybrid pepetide fragmentation and acquisition strategies and a dedicated software suite, termed XlinkX. We applied this novel XL-MS strategy to lysates of E. coli lysate and human HeLa cell lines, and to mammalian mitochondria and nuclei. In each of these studies we successfully identified thousands of cross-links. Many of the identified cross-links could be validated by mapping them on available high-resolution structures, but the data also provide information on assemblies for which no high-resolution structures are available, and even reveal new protein interaction networks.

Probing the insulin network using phosphoproteomics

Prof. David James

Insulin plays a fundamental role in regulating important physiological processes and dysfunctional insulin action is at the heart of many metabolic diseases. To probe the insulin network more deeply we utilized global unbiased phosphoproteomic analysis in cells/tissues from animals subjected to a range of perturbations. We have also developed novel visualization approaches to display the dynamic nature of this network. These studies have unveiled novel avenues of insulin action and how this network is perturbed under conditions of insulin resistance, an early risk factor for metabolic disease. We have used machine learning approaches to unravel this network yielding exciting new approaches for identifying kinase-substrate relationships beyond traditional approaches. These approaches have identified new substrates for essential kinases like Akt and mTOR. We are now coordinating these efforts with dynamic analysis of the insulin regulated metabolome. These approaches are providing completely novel insights into our understanding of complex disease.

Invited Speaker Abstract

Changing the course and impact of chronic disease: personalizing medicine

Dr. Jennifer Van Eyk

A premise of precision health is that continuous individual health monitoring will allow early disease detection, early intervention, increased quality of life and reduced health costs. Cardiovascular disease (CVD) remains a leading cause of mortality worldwide. Atherosclerosis, a primary CVD risk factor, begins early prior to clinical signs. We have focused on diagnosing early atherosclerosis by analyzing 100s of aortic and left anterior descending coronary arteries obtained from individuals (<50 years old) using DIA and DDA workflows. Using convex analysis of mixtures and differential dependent network modeling we defined the composition, network re-wiring and regulatory features associated with early atherosclerosis. Early atherosclerosis-tissue-secreted proteins were next quantified using a 72 protein MRM assay in plasma of individuals with varying degrees of CVD. Finally, using volumetric absorptive microsampling devices for remote blood collection and our robust automated sample preparation workflows we are carrying out continuous patent-centric screening of a mid-risk CVD population.

Glycoproteins: candidate urinary biomarkers in prostate cancer

Prof. Giuseppe Palmisano

GlycoProteomics Laboratory, Institute of Biomedical Sciences, University of Sao Paulo, Brazil
Prostate specific antigen (PSA) test has revolutionized the diagnosis and management of prostate cancer (PCa). PSA is tissue specific but not cancer specific. Indeed, benign prostatic hyperplasia and prostatitis can induce changes in PSA levels and lead to misdiagnosis. Due to that, novel biomarkers for PCa are needed to address the lack of specificity of PSA. Here we describe a comprehensive glycoproteomic approach to identify candidate biomarkers in urine from PCa using multiplex isobaric labelling combined with high resolution MS analysis. 1865 proteins were identified in urine. 954 intact N-glycopeptides and 965 intact O-glycopeptides were quantified in the urine from prostate cancer and control individuals. A panel of differentially regulated glycopeptides was validated by parallel reaction monitoring. This study presents an integrated experimental strategy for urine glycoproteomics with special emphasis in the current computational solutions for large-scale MS/MS spectra interpretation of intact glycopeptides. Moreover, this study highlights the possibility of using glycoproteins as important targets in PCa diagnosis.

Invited Speaker Abstract

Proteomics as diagnostic tool for platelet function

Prof. Albert Sickmann

More than 130 years ago, it was recognized that platelets are key mediators of thrombosis and hemostasis. Nowadays, it is established that platelets participate in additional physiological processes and contribute to the genesis and progression of cardiovascular diseases. Anti-platelet treatment is of fundamental importance in combatting functions/dysfunction of platelets in the pathogenesis of cardiovascular and inflammatory diseases. Owing to their anucleate nature platelets have only limited de novo protein synthesis and (dys)function of platelets is likely to be completely attributable to alterations in protein expression patterns and post-translational modifications. Consequently, platelet proteomics will represent an invaluable tool for characterizing the fundamental processes that affect platelet homeostasis and thus determine the roles of platelets in health and disease.
Combining elaborate protocols for platelet isolation from fresh blood donations in conjunction with quantitative mass spectrometry, our recent data indicate that the platelet proteome, representing the complete set of expressed proteins, comprises approximately 5,000 proteins and is remarkably stable between different healthy individuals. The protein composition and the phosphorylation patterns of platelets will be useful to understand certain activation/inhibition states and therapeutic interventions. In particular, quantitative phosphoproteomic studies will pave the way for a refined understanding of platelet properties. The detection of candidate proteins from different pathways in clinical samples as biomarker for platelet activity is further refined with the objective to achieve DIN ISO 17025 accreditation.

Sugars and proteins: towards a synthetic biology

Prof. Ben Davis

Our work studies the interplay of biomolecules – proteins, sugars and their modifications. Synthetic Biology's development at the start of this century may be compared with Synthetic Organic Chemistry's expansion at the start of the last; after decades of isolation, identification, analysis and functional confirmation the future logical and free-ranging redesign of biomacromolecules offers tantalizing opportunities. This lecture will cover emerging areas in our group in chemical manipulation of biomoleclules with an emphasis on new bond-forming and -breaking processes compatible with biology:
(i) New methods: Despite 90-years-worth of non-specific, chemical modification of proteins, precise methods in protein chemistry remain rare. The development of efficient, complete, chemoand regio-selective methods, applied in benign aqueous systems to redesign and reprogramme the structure and function of biomolecule both in vitro and in vivo will be presented.
(ii) 'Synthetic Biologics' and their applications: biomimicry; functional recapitulation; effector [drug/agrochemical/gene/radio-dose] delivery; selective protein degradation; inhibitors of pathogen interactions; non-invasive presymptopmatic disease diagnosis; probes and modulators of in vivo function.

Developmental proteomics: the importance of age specifi c diff erences in the human plasma proteome

Prof. Vera Ignjatovic

Developmental proteomics: the importance of age specific differences in the human plasma proteome
Human plasma proteomics studies have to date focused largely on: specific disease settings, detecting the highest number of proteins, as well as the effect of therapeutics on plasma protein expression. The majority of these studies have been adult-based, with very few studies and hence limited knowledge of the plasma proteome in neonates, infants and children. Developmental Proteomics, with a focus on age-specific differences in the human plasma proteome can be extremely useful in providing much needed knowledge, particularly as proteomics edges towards clinical applications/diagnostics. This presentation will outline the importance of unraveling age-based differences in the human plasma proteome.

Invited Speaker Abstract

Metaproteomic tools to study individual human gut microbiota

Prof. Daniel Figeys

Host–microbiota interactions have been linked to a growing list of diseases, including inflammatory bowel diseases (IBD), obesity, etc.. We are interested in studying the molecular changes that occurs in host and microbiota during gut dysbiosis, the effect of diet and to develop screening techniques for microbiota. To date our understanding of the microbiota has been driven by Next-generation sequencing (NGS). In contrast, the application of metaproteomics primarily due to the the lack of easy-to-use metaproteomic computational platforms that can handle the very large microbial genomic databases. We will first report on the development of a metaproteomics protein identification platform called Metalab and its application for deep metaproteomic analysis. Then we will report on RapidAIM, an ex vivo assays to study the effects of drugs on the human gut microbiota. Series of compounds can be screened against individual microbiota allowing the characterisation of the microbiota changes induced by the compounds.

Invited Speaker Abstract

Prospective from Taiwan cancer moonshot to address unmet clinical needs

Prof. Yu-ju Chen

The different genetic background and environmental factors contribute to unique features of cancers in diversity of population (e.g. Taiwan/Asian), awaiting full delineation of genomic-to-proteomic network to identify the fundamental drivers of cancer and the individual responsiveness and failure to therapy. With the aim of accelerating the progress toward prevention, control and treatment for cancer, Taiwan joined the global effort of Cancer Moonshot initiated by the US to apply proteogenomics as a precision approach to delineate the connection of genomic abnormalities and protein alteration in individual cancer patient's tissues. Subsequently, the Cancer Proteogenomics Moonshot project was included by Board of Science and Technology in Taiwan as an action plan under the "Biomedical Industrial Innovation Program", a newly launched roadmap in late 2016 to build Taiwan as Asia-Pacific hub for biomedical research and development. In this talk, I will present how this project stimulates academia-government-industry collaboration and challenges as well as our expectation to map the pathway for next generation precision medicine to address unmet clinical needs in Taiwan/Asia.

Invited Speaker Abstract

A dream: from a protein’s sequence to its structure - modern structural proteomics techniques

Prof. Christoph Borchers

We present here an integrated experimental and computational approach for de novo protein structure determination in which short-distance crosslinking data is incorporated into rapid discrete molecular dynamic (DMD) simulations as constraints, reducing the conformational space and achieving the correct protein folding on practical time scales. We tested our approach on myoglobin and FKBP — models for alpha-helix and beta-sheet rich proteins, respectively — and found that the lowest-energy structures obtained were in excellent agreement with the crystal structure and with hydrogen/deuterium exchange, surface modification, and long-distance crosslinking validation data. Our approach is readily applicable to other proteins with unknown structures.

Invited Speaker Abstract

Studying protein structural changes on a proteome-wide scale in health and disease

Prof. Paola Picotti

Protein structural changes induced by external perturbations or internal cues can profoundly influence protein activity and thus modulate cellular physiology. Mass spectrometry (MS)-based proteomic techniques are routinely used to measure changes in protein abundance, post-translational modification and protein interactors, but much less is known about protein structural changes. In my talk, I will present a recently developed structural proteomics method that enables analysis of protein structural changes on a proteome-wide scale and directly in complex biological extracts. The approach relies on the coupling of limited proteolysis (LiP) tools and MS. LiP-MS can detect subtle alterations in secondary structure content, larger scale movements such as domain motions, and more pronounced transitions such as the switch between folded and unfolded states. I will describe how the method can be applied to the study of cellular pathways, resulting in the discovery of novel regulatory events and discuss the strength and limitations of the approach.

Integrated ‘omics and their role in personalized healthcare now and in the future

Prof. Alain Van Gool

We are now in midst of the era of personalized medicine: provide the right drug to the right patient at the right dose at the right time. Recently, society is driving this even more upstream towards early diagnosis of non-healthy states and prevention of disease using mHealth and eHealth tools, changing the model to personalized healthcare. Key drivers of the personalized medicine and healthcare models are molecular biomarkers. As human systems are extremely complex, multiple biomarkers are needed to reflect the state of the biological system. The various ‘omics technologies are powerful approaches to identify and validate such biomarkers, in combination with cellular and clinical researchers, high quality biobanks and data scientists, to name a few. Although the ‘omics platforms have not fulfilled the great expectations of the past decades regarding impact on healthcare, several promising developments suggest major breakthroughs in the years to come. Examples will be discussed, along with new initiatives to organize technology infrastructures to drive personalized medicine and health research.

Proteomic exploration of the scope, dynamics, and stoichiometry of lysine acetylation

Prof. Chuna Choudhary

Lysine acetylation is a key regulatory posttranslational modification. Function of lysine acetylation is most extensively studied in the context of epigenetic regulation of gene transcription via acetylation of histones. Recent mass spectrometry (MS)-based proteomic studies have greatly expanded our knowledge of this modification. Our laboratory is using MS-based quantitative proteomics to map the scope of acetylation in diverse organisms and to investigate its dynamic regulation in response to genetic and environmental perturbations. Furthermore, we developed novel proteomic methods to accurately quantify stoichiometry of acetylation on a proteome-wide scale. We showed that acetylation can occur through both enzymatic and non-enzymatic mechanisms, and our results suggest an important function of sub-cellular compartmentalization in the evolution of acetylation signaling in eukaryotes. Our systems-wide investigations provide important insights in to the mechanisms, dynamics, evolution, and specificity in acetylation signaling. I will discuss our recently published and ongoing efforts in understanding acetylation signaling.

Invited Speaker Abstract

Managing health and disease using big data

Dr. Michael Snyder

Managing Health and Disease Using Big Data
Understanding health and disease requires a detailed analysis of both our DNA and the molecular events that determine human physiology. We performed an integrated Personal Omics Profiling (iPOP) of 100 healthy and prediabetic participants over three years including periods of viral infection as well as during controlled weight gain and loss. Our iPOP integrates multiomics information from the host (genomics, epigenomics, transcriptomics, proteomics and metabolomics) and from the gut microbiome as well as wearable information. Longitudinal multiomics profiling reveals extensive dynamic biomolecular changes occur during times of perturbation, and the different perturbations have distinct effects on different biological pathways. Wearable data also adds unique early detection information. Overall, our results demonstrate a global and system-wide level of biochemical and cellular changes occur during environment exposures and omics profiling can be used to manage health.

How proteomics impacts projects in pharma

Prof. Hanno Langen

Proteomics technologies can be used in several key aspects of modern drug development:

- to identify drug targets after phenotypic screens
- to provide biomarkers and diagnostic tests
- to provide better, safer and more efficacious treatments
- identification of pharmaco-dynamic markers

Several examples in this context will be presented. Antibody based and Mass spec based technologies are key for the major Proteomics applications. One example will be chemical proteomics which is a mass spectrometry (MS)-based, affinity chromatography approach that uses immobilized small molecules as bait to capture and identify interacting protein complexes from an entire proteome. This technique has been successfully applied, for exercises such as exploring their mode of action, and on natural products for target identification. A new area is targeted quantitative proteomics using the SRM approach. I will show examples where we were we applied a 250 plex quantitative assay in neuronal stem cells. The discovery of biomarkers and the validation of them in clinical samples – and the hurdles to overcome this issue will be presented.

Proteomics for signaling and clinical studies

Prof. Matthias Mann

Mass spectrometry-based proteomics has become an indispensable part of biochemical science and is used in a wide variety of contexts (Aebersold and Mann, Nature, 2016). In this talk, I will focus on the latest improvements and application of phosphoproteomics as well as clinical proteomics. Building on the 'EasyPhos' method developed recently (Humphrey et al. Nat. Biotech, 2015), we are now studying complex signaling events in vivo. For instance, application of EasyPhos to the circadian rhythm has revealed that a large percentage of the phospho-proteomics is coordinately regulated during the day and night cycle, and many of the target sites appear to fine tune the metabolic machinery (Robles et al. Cell Metabolism, 2017). Ongoing work in our laboratory has unraveled signaling downstream events downstream of opioid receptors in the brain in the context of analgesia and addiction.
The plasma proteome represents one of the remaining challenges of proteomics and its efficient analysis would be of huge importance to medical diagnostics. We recently described the Protein Correlation Profiling approach, which allows us to study the plasma proteome rapidly in a wide range of conditions (Geyer et al. Cell Systems 2016). We have now drastically increased the protein coverage and applied it to a number of clinical studies (Geyer et al. MSB 2016). The talk will summarize what we have learned from these studies in the particular context of metabolic disease and about the nature of the plasma proteome in general.

Deciphering the glycoproteome: a small step towards understanding the complexity

Prof. Pauline Rudd

Alterations in glycosylation are common in physiological and pathological processes. Glycan structures are, in the first instance, controlled by genes, however the complex pathways, systems, environment and epigenetic factors that regulate their expression may provide a further mechanism for fine tuning physiological responses by diversifying the glycans and the functions of the proteins to which they are attached. Determining the impact of post-genomic factors on glycosylation processing is a relatively underexplored field and has led us to develop new automated multiplexed technologies and bioinformatics that can match the output from other –omics platforms. This talk will focus on our recent attempts to explore an integrated glycobiology approach to cancer that links the glycome to the proteome, transcriptome, metabolome, lipidome and genome. This has enabled us to gain a deeper insight into some of the pathways involved and is a step towards precision medicine.

Invited Speaker Abstract

The cell-based human proteome project: mapping human proteins and their complexes with complete molecular specifi city

Mr. Neil Kelleher

The Cell-Based Human Proteome Project seeks to define and map detectable proteoforms all throughout the human body, allowing us to improve proteomics technology and revolutionize our understanding of how wellness and disease manifests at the level of protein composition in human biology. Building on the successes of the Human Genome Project, the existing drafts of the Human Proteome, and the Human Cell Atlas, the Cell-Based Human Proteome Project (CB-HPP) also offers a transformative next step in our ability to understand and improve human health through more precise mapping of protein compositional space. In this brief talk, Neil will share the vision ahead for the CB-HPP, including pilot level progress, supported by the Paul G. Allen Family Foundation (the Human Proteoform Atlas) and the Consortium for Top Down Proteomics, in clinical research in areas such as organ transplantation, heart disease, and cancer. The core of the CB-HPP involves mapping of 1 billion proteoforms throughout the human body for $1 each, and a brief TEDx talk describing the project is available here: https://www.youtube.com/watch?v=hHJxMnq51KU

Variability in mass spectrometry-based quantifi cation of clinically relevant drug

Prof. Per Artursson

Variability in mass spectrometry-based quantification of clinically relevant drug transporters and drug metabolizing enzymes in the human liver – impact on pharmacokinetic and toxicokinetic predictions
Per Artursson, Dept of Pharmacy, Uppsala University
Many different methods are used for mass-spectrometry based protein quantification in pharmacokinetics and systems pharmacology. It has not been established to what extent the results from these various methods are comparable. Here, we compared six different mass spectrometry-based proteomics methods by measuring the expression of clinically relevant drug transporters and metabolizing enzymes in human liver. Protein quantities obtained from label-free and targeted methods using whole tissue lysates were in general in good agreement. Methods using subcellular membrane fractionation gave incomplete enrichment of targeted proteins. When these quantified proteins were adjusted to levels in whole tissue lysates, they were on average four-fold lower than those quantified directly in whole tissue lysates. These differences were propagated into differences in pharmacokinetic predictions of hepatic drug clearance in man. In conclusion, caution is needed when applying quantitative proteomics data obtained with different methods in pharmacokinetic and toxicokinetic modeling.

Invited Speaker Abstract

Pattern of MAP kinase phosphatase, DUSP1, in human obesity, diabetes and

Dr. Ali Tiss

Dual-specificity protein phosphatase 1 (DUSP1) is regulating the activity of MAP Kinases and is implicated in energy expenditure. We investigated the expression pattern of DUSP1 in normal-weight, non-diabetic and diabetic obese human subjects using subcutaneous adipose tissue and blood. A strong correlation of DUPS1 levels was observed with obesity indicators, inflammatory and metabolic markers which were then modulated by a 3-months moderate physical exercise intervention. We further investigated DUSP1 circulating levels in selected phenotypes of human cardiovascular disease (CVD) patients in a separate human cohort. CVD patients displayed higher levels of DUSP1 whereas no difference was seen in hsCRP levels when compared to their matched controls. Our results suggested that CVD patients are experiencing ongoing inflammation and that circulating DUSP1 might be considered as potential marker for residual risk and future CVD events

Invited Speaker Abstract

Microvesicle-based identifi cation of cancer biomarkers for early detection of ovarian

Prof. Tamar Geiger

Microvesicle-based identification of cancer biomarkers for early detection of ovarian cancer

Gerogina Barnabas1, Michal Harel1, Keren Bahar-Shany2, Keren Levanon2 and Tamar Geiger1

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Mass spectrometry-based identification of cancer biomarkers in body fluids, has great potential to develop into non-invasive diagnostic tests. However, the tremendous dynamic range of proteins in the plasma hampers identification of low abundant biomarkers. To overcome this challenge we isolate plasma microvesicles, which are largely devoid of these highly abundant proteins. Using this method, we identified 3638 plasma-derived proteins in triplicate runs, and quantified disease biomarkers in cancer-patient plasma specimens1. We applied this technology to early detection of ovarian cancer, but rather than examining plasma, we analyzed uterine lavage fluid, which has the potential to contain tumor markers at an early stage. Proteomic analysis of purified microvesicles from 134 samples identified >8000 proteins, and yielded a minimal diagnostic signature of 21 proteins, which provided high specificity (91%) and correctly identified stage IA lesions. Altogether, this platform enables high throughput identification of biomarkers to allow early diagnosis and personalized treatment.

Invited Speaker Abstract

The human immunoepeptidome project – accelerating the development of

Dr. Michal Bassani

In the first part of my talk I will present the recently launched HUPO Human Immuno-Peptidome Project (HUPO-HIPP). The long-term goal of this project is to map the entire repertoire of peptides presented by human leukocyte antigen (HLA) molecules using mass spectrometry technologies. I will summarize the discussions held during the 1st HUPO-HIPP workshop (May 2017) and the current ongoing activities of this initiative.
In the second part of my talk I will present how my lab develops and applies advanced in-depth high-throughput mass-spectrometry based immunopeptidomics for the development of personalized cancer vaccines. We have shown that MS analysis of HLA-I binding peptides (HLAp) eluted from tissue samples is a promising approach to discover the actual in-vivo presented neo-antigens; yet, it is applicable to a small fraction of samples due to the large amount of biological sample that is required. Still, the massive amount of HLAp data acquired while hunting down the neo-antigens is highly valuable. We show that by taking advantage of co-occurring HLA-I alleles across dozens of immunopeptidomics datasets we can rapidly and accurately identify HLA-I binding motifs and map them to their corresponding alleles without any a priori knowledge of HLA-I binding specificity. Consequently, training HLA-I ligand predictors on refined motifs significantly improves the identification of neoantigens.

Immunoassays in multiplex for biomarker discovery and validation

Dr. Thomas Joos

Bridging the Gap in Oncology Diagnostics: Converting Omic Data into Clinically Relevant Assays
Array-based assay systems allow the analysis of hundreds of molecular parameters in a single experiment. Within the last decade protein microarray technologies achieved robust analytical performance and enable to screen for a multitude of parameters using minimal amounts of sample material.
There are a variety of technologies and methods available to address assay requirements in terms of covering analyte concentration, sample variety and sample origin. An overview of classical and emerging immunoassays ranging from sandwich-, mass spectrometry-, bead array-, and digital array-based immunoassays will be presented. Advantages of the different methods will be demonstrated by addressing biological issues for serological screening approaches and drug-induced injury biomarker research, toxicology and oncology.

Medical and biological insights derived from SOMAscan

Dr. Larry Gold

We have spent nearly twenty years developing a rapid proteomic scanning platform (called SOMAscan) that currently quantifies more than 5,000 human proteins simultaneously, and which will eventually scale to the entire human proteome (~20,000 unique proteins). The current median limit of detection for SOMAscan is 40 fM, and the platform is run with neither depletion nor chromatography. SOMAscan has an average CV of ~5% for the 5,000-plex. To date, we have analyzed more than 125,000 samples derived from human serum, plasma, urine, and tissue extracts, and we have begun to build a series of insights into biology that could alter the ways we think about maintaining health and wellness.

I will share some of those insights today. I will also provide some new information on SOMAmers, the protein-binding reagents that make SOMAscan so powerful. I will show that SOMAmers are capable of extraordinary discrimination individually and in the full assay, where they are able to quantify proteins in the presence of other nearly identical proteins (e.g., GDF-11 and GDF-8). Finally, I will talk about ways that clinical researchers can access this powerful technology through collaborations with SomaLogic.

Democratizing cancer proteogenomic data

Prof. Bing Zhang

Large-scale cancer omics projects such as TCGA and CPTAC have produced a vast amount of genomic and proteomic data. To fully realize the potential of these data, we will need to make them directly available and usable to the entire cancer research community. In this talk, I will introduce two tools for democratizing cancer proteogenomic data: LinkedOmics and PepQuery. LinkedOmics (http://www.linkedomics.org) is a web platform for exploring associations between different types of molecular and clinical attributes, comparing associations discovered from different omics platforms or tumor types, and interpreting identified associations in the context of biological pathways and molecular networks. PepQuery is a peptide-centric search engine that makes mass spectrometry proteomics data directly available for validating novel peptides predicted from genomic studies.

Translating a trillion points of data into therapies, diagnostics and new insights into desease

Atul Butte

There is an urgent need to take what we have learned in our new "genome era" and use it to create a new system of precision medicine, delivering the best preventative or therapeutic intervention at the right time, for the right patients. Dr. Butte's lab at the University of California, San Francisco builds and applies tools that convert trillions of points of molecular, clinical, and epidemiological data — measured by researchers and clinicians over the past decade and now commonly termed "big data" — into diagnostics, therapeutics, and new insights into disease. Several of these methods or findings have been spun out into new biotechnology companies. Dr. Butte, a computer scientist and pediatrician, will highlight his lab's recent work, including the use of publicly-available molecular measurements to find new uses for drugs including new therapies for autoimmune diseases and cancer, discovering new druggable targets in disease, the evaluation of patients and populations presenting with whole genomes sequenced, integrating and reusing the clinical and genomic data that result from clinical trials, discovering new diagnostics include blood tests for complications during pregnancy, and how the next generation of biotech companies might even start in your garage.

Cell free methods for producing protein microarrays

Dr. Joshua Labaer

Self-assembling protein microarrays arrays can be used to study protein-protein interactions, protein-drug interactions, search for enzyme substrates, and as tools to search for disease biomarkers. In particular, recent experiments have focused on using these protein microarrays to search for autoantibody responses in cancer patients. These experiments show promise in finding antibody responses that appear in only cancer patients. New methods using click chemistry-based reagents also allow the application of these arrays for discovering new substrates of post translational modification.

Invited Speaker Abstract

Development of a large scale integrated platform for clinical proteomics and drug target discovery

Prof. Tony Whetton

Precision medicine is a key objective in improving healthcare, thus enhancing the speed and throughput for companion diagnostic, target and biomarker discovery is essential. The use of innovations in MS-based technologies offers a high capacity throughput proteomic profiling for clinical biochemistry purposes that can be industrialised, as has been achieved at the Stoller Biomarker Discovery Centre. For example, markers of risk in ovarian cancer have been investigated using a SWATH MS approach. We have also identified potential new curative strategies for chronic myeloid leukaemia and polycythaemia vera. Combined with validation platforms, this approach offers a quicker route to mechanistic detail/drug targets plus biomarkers for risk and stratification. Linkage of proteomic data to electronic health records through safe haven health informatics allows the integration of complex molecular phenotyping with endotypic data. By scaling up proteomics platforms and adding pathology expertise, we reduce the time for biomarker development for patient benefit.

Development of liquid-biopsies for personalized care in prostate cancer

Prof. O. John Semmes

Prostate cancer (PCa) is a major health problem in males in the United States. The diversity and heterogeneity of PCa is well appreciated with considerable variation in clinical course ranging from asymptomatic disease to a rapidly progressing fatal malignancy. Current risk-stratification strategies lack adequate sensitivity and specificity to discriminate aggressive from indolent diseases. Optimal management of men with prostate cancer requires clinically robust biomarkers for the early detection of aggressive disease, to monitor those on an active surveillance program and to guide treatment decisions following diagnosis. Cognizant of these needs, our research efforts are focused on the development of protein-based biomarkers derived from post-DRE urine as novel liquid biopsies to assist in the early detection of invasive (pT3) disease. We will discuss our current biomarker development achievements and activities aimed at successfully navigating assay validation.

Invited Speaker Abstract

Proteomics and immunoproteomics of gram-positive bacteria in host-pathogen

Dr. Frank Schmidt

S. aureus related diseases range from mild to severe infections. In general, proteome analysis approaches using data dependent acquisition (DDA) are known to provide lower reproducibility and comprehensiveness when compared to data-independent acquisition (DIA). In this study, a DIA spectral library was generated and benchmarked with a well characterized biological standard and used for the analysis of S. aureus interacting with S9 and murine cells. Protein analysis revealed up-regulations in oxidative stress, cell wall assembling and down-regulations in dNTP synthesis or biosynthetic activity. DIA analysis allowed a deep insight in the pathogen-host adaption and revealed many infection specific proteins which we further overexpressed as recombinant proteins to investigate the antibody response of S. aureus carrier or non-carrier or of patients suffering on sepsis. We further profiled their blood proteomes by DIA in order to find new candidates for diagnosis and prognosis.

Invited Speaker Abstract

Systems biology of oncogenic kinase signalling

Dr. Pedro Cutillas

Kinase inhibitors are revolutionizing the way most tumour types are treated. However, not all cancer patients respond to these compounds to the same extent, and relapse limits their efficacy. The work in my group aims to understand why some tumours respond to targeted therapies while others are resistant to the same treatments. Using label-free phosphoproteomics and computational approaches for inferring kinase activity from phosphoproteomics data, we found that the activities of pathways acting in parallel to PI3K determine whether or not primary leukaemia cells may respond to PI3K inhibitors. Using similar approaches, we have recently found that specific combinations of pathway activities explain the mode of action of MEK and FLT3 inhibitors in leukaemia. These data suggest that technology for measuring the signalling network as a whole (rather than just the pathway that is being targeted) may be able to predict clinical sensitivity to signalling inhibitors with high accuracy.

What controls the three dimensional proteome?

Prof. Kathryn Lilley

Proteins can adopt multiple functions, depending on the isoform expressed, and variations in their sub-cellular location, binding partners and post-translational modifications. Such differential control significantly increases the functionality of the proteome. The processes governing these features are highly dynamic and their aberrant control is implicated in many diseases.
We have developed methods to determine the three dimensional organisation of the proteome in eukaryote cells in a single experiment with high resolution (Mulvey et al, Nature Protocols (2017)). We have applied this method to many different biological systems, including mapping protein relocalization upon perturbation. Intriguingly, we consistently observe that up to half the proteome cannot be discretely assigned to a single localisation. This observation has been recently supported by comparing two very different approaches to map proteins to their subcellular niches (Thul et al, Science (2017)).
In this presentation I will discuss factors controlling protein location and the interplay of the metabolism and signalling mechanisms on the spatial proteome.

Invited Speaker Abstract

The ups and downs of protein expression regulation

Dr. Christine Vogel

Gene expression is regulated by four major processes: transcription, translation, and RNA and protein degradation. These processes are adjusted, in different ways, when the cells respond to a stimulus. Many pathways are known, but their precise interaction over time is not well understood. In our lab, we use multiple time series datasets — on protein and mRNA expression changes and changes in the binding of ribosomes and other proteins — in combination with mass action models and other approaches to disentangle the contributions of the different levels of regulation and generate hypotheses on regulatory mechanisms. We focus on yeast and mammalian cells responding to stress of the endoplasmic reticulum, but have expanded these studies in a variety of directions.

Translating MS protein assays into the clinic: potential of post-translationally modified proteins for clinical MS protein tests

Dr. Dobrin Nedelkov

Protein MS assays are forecasted to be the next-generation tests for precise and enabling measurement of clinical protein biomarkers. But in the 30 years since the MALDI and ESI MS invention, only a dozen protein MS tests have been translated into clinical laboratories. Analytical performance requirements have been in place for some time, along with small molecules MS clinical tests precedents, so it seems that key clinical and economic drivers have not been met for their adoption. Even when MS approaches result in new protein biomarkers discovery, enzymatic immunoassays oftentimes replace MS in clinical lab tests. One way to drive translation and adoption of MS protein tests is to target protein features that could only be detected with MS – such as post-translational modifications (PTMs) – thus generating both content and demand. Discussed in this presentation will be some viable PTM protein targets and the path forward for these clinical MS protein tests.

Invited Speaker Abstract

The proteome in context

Ruedi Abersold

Advances in discovery proteomics have made it possible to map the protein contents of cells to saturation, at least at the resolution of expressed loci. To gain new biological or clinical insights, to date, the thus identified proteins have been largely treated as independent entities. However, proteins generally function in context with other biomolecules in the form of macromolecular assemblies. To understand how the proteome as a whole controls, coordinates and catalyzes most biochemical functions of a cell, it is therefore essential to also determine its spatial and temporal organization.

In this presentation we will discuss emerging computational and quantitative proteomic techniques to determine the organization of the proteome and to extract functional information from the results. The ultimate goal of this work is to reach a comprehensive understanding how the proteome, considered as a complex system, reacts to genetic or external perturbations and thus determines cellular and organismic phenotypes.

Invited Speaker Abstract

Novel roles of PRMT1-mediated protein-methylation in DNA damage response and miRNA biogenesis revealed by MS-proteomics

Dr. Tiziana Bonaldi

The role of non-histone protein arginine (R) methylation has been established in almost every aspect of cellular biology and its deregulation correlates with diverse pathological conditions, including cancer (1). Building on our recent evidence of the recurrence of this post translational modification (PTM) both on proteins involved in the DNA damage response (DDR) and on several subunits of the Large Drosha Complex (LDC) (2), we developed two studies aiming at the molecular and functional characterization of R-methylation in these biological processes.

I will present recent unpublished data on the systematic identification of PRMT1 substrates, through an analytical strategy combining heavy-methyl and standard SILAC, followed by ad hoc data analysis pipeline. Through quantitative profiling of the methyl-proteome dynamics during the DNA damage response (DDR) we showed that PRMT1 accumulation on chromatin during DDR correlates with global R-methylations changes of both histone and non-histone proteins and that PRMT1-dependent methylation changes massively affect the LDC structure and function, causing the overall impairment of microRNA synthesis.

Invited Speaker Abstract

A glimpse of CNHPP: a proteomic landscape of diff use-type gastric cancer

Prof. Jun Qin

China is at the leading edge of catching the wave of rapid development in proteomics. Chinese Human Proteome Project (CNHPP) is a large undertaking at dissection of the proteomic landscape of cancer. Diffuse-type gastric cancer (DGC) accounts for 30% of Gastric cancer (GC), with the worse clinical outcomes. Here we describe the first proteomic landscape of diffuse-type gastric cancer. Based on proteome profiling data of 84 DGC samples, DGC can be subtyped into 3 major classes that exhibit distinct proteome features. Importantly, the 3 subtypes are correlated with distinct clinical outcomes and adjuvant chemotherapy effect. To facilitate potential clinical applications, we further developed a simplified classifier, which is able to distinguish three DGC subtypes with 7 protein markers. Integrated analysis of proteome and targeted exome sequencing data revealed a loose connection between the genome and the proteome. It is striking that for many mutational cancer driver genes, their gene products were never detected in the gastric proteome, which raises a serious concern about using DNA sequencing to predict protein expression and highlights the necessity of measuring proteins directly for precision medicine. We also nominated several druggable candidates for developing therapy against DSG based on the altered DGC proteome and the association with patients' overall survival.

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Quantitative Proteomics To Investigate the Effects of a pMRTP And Smoking Cessation On Mouse Lung

Ms. Sophie Dijon[1], Ashraf Elamin[1], Bjoern Titz[1], Celine Merg[1], Marcel Geertz[1,3], Thomas Schneider[1], Florian Martin[1], Walter Schlage[1], Stefan Frentzel[1], Fabio Talamo[1], Emilija Veljkovic[2], Nikolai V. Ivanov[1], Patrick Vanscheeuwijck[1], Manuel C. Peitsch[1], Julia Hoeng[1]
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Smoking causes many serious diseases, including lung cancer and chronic obstructive pulmonary disease (COPD). Within our systems toxicology framework, we are assessing whether potential modified risk tobacco products (MRTP) can reduce smoking-related health risks compared to conventional cigarettes.
In this study, we evaluated the effects of cigarette smoke (CS) from a standard reference cigarette (3R4F) and aerosol from a prototype MRTP (pMRTP) on C57BL/6 mice. They were exposed to high concentrations of 3R4F CS, aerosol from RRPs or filtered air (Sham) for up to 7 months. The assessed pMRTP utilize "heat-not-burn" technologies and were matched in nicotine concentrations to the 3R4F CS. After 2 months exposure to CS, cessation and switching groups were further exposed for up to 6 months to fresh air, or to the pMRTP, respectively. Isobaric tag-based quantitative proteomics (iTRAQ®) analyzed by LC-MS/MS and 2D-PAGE/MALDI MS/MS were used to assess the exposure effects on the mouse lung proteome. We evaluated to what extent 2D-PAGE/MALDI MS/MS can complement the iTRAQ results. Selected differentially expressed proteins identified by both LC-MS/MS and 2D-PAGE/MALDI MS/MS approaches were further verified using reverse-phase protein microarrays (RPPA).
LC-MS/MS captured the effects of CS on the lung proteome more comprehensively than 2D-PAGE. However, an integrated analysis of both proteomics data sets showed that 2D-PAGE data complement the LC-MS/MS results by supporting the overall trend of lower effects of pMRTP aerosol than CS on the lung proteome. Furthermore, the RPPA results were in agreement with previous results. Biological effects of CS exposure supported by both methods included increases in immune-related, surfactant metabolism, proteasome, and actin cytoskeleton protein clusters.
Overall, while 2D-PAGE has its value, especially as a complementary method for the analysis of effects on intact proteins, LC-MS/MS approaches will likely be the method of choice for proteome analysis in systems toxicology investigations.

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New Proteomics Capabilities using SUPER SLIM High Resolution Ion Mobility-Mass Spectrometry

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Background:
Advances in MS-based proteomics are increasingly driven by the sophistication of ion manipulations prior to actual mass analysis, and also often limited in their speed wen done in conjunction with the use of liquid chromatography (LC) separations. In this regard, ion mobility (IM) separations after ionization are of interest not only for the enhanced separation power, allowing more complete and effective proteome characterization, but also for the additional structure-related information (collision cross sections) derived from these separations, and that aids identification. The benefits of IM separations increase as separation power increases, but their resolution has been modest compared to LC, and even the modestly higher resolution IM separations demonstrated to this point have only been achieved in conjunction with significant ion losses.

Methods:
The focus of this presentation will be new developments based upon Structures for Lossless Ion Manipulations (SLIM) that address many current challenges for MS-based 'omics' applications, and that also provide new capabilities for MS.

Results:
New developments based upon the rapid evolution of SLIM will be described, including their use for development of serpentine ultra-long path with extended routing (SUPER) IM devices providing much higher IM resolution than previously possible, and enabling previously problematic separations. Importantly, this development presents the potential for eliminating the need for other on-line separations with MS. Examples of applications include the separation and quantification of D-amino acid containing peptides, a significant current 'blind spot' for MS-based proteomics.

Conclusions:
A large advance in the throughput and quality of proteomics measurements is shown based upon a new implementation of SLIM IM-MS.

Keywords:
ion mobility-mass spectrometry, resolution, throughput, sensitivity, SLIM

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SPECTRUM: A MATLAB toolbox for identifying proteins from top-down proteomics data

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Proteins play a central role in regulating cell life, and proteomic anomalies are known to give rise to a variety of disorders including cancer and diabetes. The advent of high-resolution proteomics has facilitated accurate protein identification and quantification thus creating avenues for improved disease characterization and diagnoses. Proteomics commonly employs “Bottom-Up” (BUP) and “Top-Down” (TDP) protocols which analyze enzymatically digested and whole proteins, respectively. TDP has an edge over BUP in that it provides for (i) measurement of accurate intact protein mass, (ii) a high sequence coverage and (iii) identification of post-translational modifications (PTM). Since top-down proteomics is a maturing protocol, limited software support is available for associated data analysis in the form of search engine pipelines and algorithms. In this work, we propose “SPECTRUM”, a MATLAB toolbox for analyzing top-down proteomics data. SPECTRUM is free, open source and open architecture toolbox that provides an intuitive search environment for identifying proteins. Its salient features include: (i) multiple data file format support including extensible markup language (mzXML), Mascot Generic Format (MGF) and flat text files, (ii) intensity weighted sliding window protocol for intact protein mass tuning, (iii) de novo peptide sequence tag extraction and its scoring (using fragment intensity, accumulated mass error and tag frequency), (iv) identification of PTMs using statistically inferred binding sites, (v) abundance weighted in silico spectral comparison, (vi) a multifactorial additive scoring scheme employing coefficient weighted constituent scores and (vii) a set of WYSIWYG graphical user interfaces (GUIs) built using MATLAB GUIDE providing access to the aforementioned functionalities. Taken together, SPECTRUM is a freely available top-down protein identification toolbox that can be employed in development and benchmarking of novel top-down proteomics algorithms. The proposed toolbox can also assist proteomics instructors in classrooms by providing them a platform for educational and training purposes.

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Investigation of the lymphatic malformation cystic fluid and plasma proteomes

Dr Zerina Lokmic[1,2,3], Mrs Dana Pascovici[4], Mrs Thiri Zaw[4], Dr Xiaomin Song[4], Dr Elhamy Bekhit[3], Prof Anthony Penington[3], A/Prof Mark Molloy[4], Ass. Prof. Vera Ignjatovic[1,2]

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Background
Human lymphatic malformations are slow-flow vascular anomalies caused by a somatic mutation in PIK3CA gene arising in the endothelial cells lining lymphatic malformation vessels. Unlike in normal lymphatic vessels, the lymph accumulating in the lymphatic malformation cysts is mostly stagnant as the cysts are dysfunctional and do not effectively empty into the lymphatic vascular system. This study aimed to examine the lymphatic malformation cystic fluid (CF) proteome and compare it to matched plasma proteome to identify differentially expressed proteins and the corresponding cellular pathways where these proteins might play a role.

Methods
Matched CF and matched plasma samples (n=6) were collected from children undergoing first-time sclerotherapy for the management of their lymphatic malformation. Both were investigated using SWATH-MS (5600 TripleTOF MS (Sciex) coupled to an Eksigent Ultra-nanoLC-1D system (Eksigent, Dublin, CA)).

Results
In total, 274 proteins were detected across all CF and plasma samples, with no protein being unique to CF. There were 134 differentially expressed proteins that grouped into 6 clusters of expression. These represented KEGG pathways of complement and coagulation cascades, with the most significant gene ontology functions being: protein activation cascade, complement activation, complement activation classical pathway, humoral immune response and acute inflammatory response. The 5 pathways represented were: Acute Phase Response Signalling (17.2% overlap); FXR/RXR Activation (19.0% overlap); LXR/RXR activation (22.3% overlap); Complement system (32.4% overlap) and Coagulation system (37.1% overlap).

Conclusions
This is the first study to characterise the lymphatic malformation cystic fluid proteome and to demonstrate differences in protein expression compared to the matched patient plasma proteome. These differences are likely to be a reflection of the lymphatic malformation pathology, whereby differentially expressed proteins are either the product of accumulation due to stagnation of the lymphatic malformation cystic fluid or alternatively are synthesised at a different rate by the mutated endothelium.

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Early protein biomarker discovery in urine of Walker 256 subcutaneous rat model

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Despite advances in cancer treatments, early detection of cancer is still the most promising way to improve outcomes. Without homeostatic control, urine reflects early changes in the body and can potentially be used for early cancer diagnosis. In Walker 256 subcutaneous tumor rat model, urine samples were collected at five time points corresponding to before cancer cell implantation, before tumor mass palpable, at tumor mass appearance, during rapid tumor growth, and at cachexia. The urinary protein patterns changed significantly on SDS-PAGE as the tumors progressed. The urinary proteome of tumor-bearing rats was identified using label-free quantification. Thirty dynamically changed urinary proteins during cancer progression were selected as candidate cancer biomarkers and validated by targeted proteomics. A total of 10 urinary proteins (HPT, APOA4, CO4, B2MG, A1AG, CATC, VCAM1, CALB1, CSPG4, and VTDB) changed significantly even before a tumor mass was palpable, and these early changes in urine could also be identified with differential abundance at late stages of cancer. Our study indicated that urine is a sensitive biomarker source for early detection of cancer.

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Early detection in urinary proteome for effective treatment of bleomycin-induced pulmonary fibrosis in rat model

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Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal fibrotic lung disease. With limited effective treatments available in the late stage, IPF has a very poor prognosis, with an average survival time of under 3 years. Molecular biomarkers are highly desired for IPF, especially for its early phase. Lacking homeostatic control, urine is a better biomarker source than blood for detecting small and early pathological changes. In this study, urine samples were collected from rats with bleomycin-induced 15 pulmonary fibrosis. Samples collected during slight fibrosis were used for early diagnostic biomarker identification; 11 differentially expressed proteins were identified by labeled proteome quantitation, four of which were previously reported to be associated with fibrosis. In samples during fibrosis progression, 30 differentially expressed proteins were identified as biomarkers for disease monitoring, many of which have been reported to be associated with IPF pathogenesis. Prednisone treatment was administered at different phases. It was found that prompt prednisone treatment after early diagnosis effectively inhibited lung fibrosis, whereas same treatment at late phase had very limited effects. Compared with differential proteins during lung fibrosis, change trends of 5 differential proteins were reversed after prednisone treatment, and they could serve as therapeutic monitoring biomarkers.
Urinary proteomics have been underutilized in respiratory diseases for decades. This is the first time urinary proteomics has been applied to lung fibrosis disease. Our results showed that urine proteins have the potential for early diagnosis, monitoring disease progression and reflecting treatment efficacy in IPF and probably other lung diseases. These findings may lead to better understanding of pathogenesis of pulmonary fibrosis.

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There are still proteins in rat urine after 7-day starvation

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Protein has been found in urine in all relevant studies. Why are there proteins in urine? If the protein in urine acts as a nutrient, should all proteins be reabsorbed into the blood during starvation to maintain the homeostasis of the internal environment? Are they toxic? Is there any protein in urine when the animal is starved? If the protein in urine is toxic or is discarded for the regulation of body's own cellular functions, it still must be released into the urine even when the animal is starved. Does the kidney need protein to maintain urine flow? If the protein is necessary to maintain urine flow, at least some of it should remain in the urine even after starvation. In this study, five Sprague Dawley rats were starved continuously for 7 days. The quantity, composition, and posttranslational modification of the urine proteome was studied before and after starvation. After 7-day starvation, urinary protein concentration had no significant changes, even though the serum protein concentration decreased for about 10%. Only five and thirteen urinary proteins were significantly changed in the 4- and 7-day starvation groups, respectively, compared with before starvation group. These findings indicate there were still proteins even after starvation which supports that urinary proteins may be necessary for kidney to maintain the urine flow or be toxic and discarded for the regulation of cellular functions. Not all potentially nutritious to the body will be reabsorbed and utilized. It seems that removing proteins in urine is even more important than maintain the homeostasis of the internal environment for the survival of the animals.

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Changes of Urinary Proteins in a Bacterial Meningitis Rat Model

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Urine has the potential to be a source of early biomarkers for chronic diseases because, unlike the homeostatic mechanisms of cerebrospinal fluid (CSF) or blood, urine accumulates metabolic changes from the whole body. The urinary proteome is susceptible to many factors, and identifying valuable early biomarkers for a particular condition using clinical samples is challenging. Bacterial meningitis is associated with a high degree of mortality. To avoid unnecessary drug use and reduce mortality and disability rates, there is a need to develop a non-invasive method for identifying biomarkers for the early diagnosis of bacterial meningitis. In this study, we used an animal model to mimic the pathophysiological condition and disease progression of bacterial meningitis. Urine samples were collected at day 1 and day 3 after an intraventricular injection with bacteria, and urinary proteins were digested by two different methods. All peptides were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The relative abundance of 35 urinary proteins changed, as determined by label-free quantification. Of these, 29 urinary proteins have human orthologs and several differentially expressed proteins, including alpha-1-acid glycoprotein (AAG), complement C4, alpha-1-antiproteinase (AAT), galectin-3-binding protein, fibronectin, and serotransferrin, have been reported as major biomarkers of bacterial meningitis.

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A dry method for preserving tear proteins

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Tears covering the ocular surface is an important bio-fluid containing thousands of molecules, including proteins, lipids, metabolites, nucleic acids, and electrolytes. Tears are valuable resources for biomarker research of ocular and even systemic diseases. For application in biomarker studies, tear samples should ideally be stored using a simple, low-cost, and efficient method along with the patient's medical records. For this purpose, we developed a novel Schirmer's strip-based dry method that allows for storage of tear samples in vacuum bags at room temperature. Using this method, tear protein patterns can also be preserved. Liquid chromatography-mass spectrometry/mass spectrometry analysis of proteins recovered from the dry method and traditional wet method showed no significant difference. Tear contains proteins enriched in 44 tissues. When tissues or organs are altered under physiological or pathophysiological conditions, tears may reflect the changes occurring in different tissues and organs. This dry method facilitates sample transportation and enables the storage of tear samples on a large scale, increasing the availability of samples for studying biomarker of diseases in tears.

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Identification of candidate prostate cancer biomarkers in urinary extracellular vesicle proteins by label-free LC-MS/MS-based proteomics.

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Background:
Prostate cancer (PCa) is the most common cancer in males in Western countries. Extracellular vesicles (EVs) secreted by the prostate (cancer) cells can be isolated from urine, of which the collection is minimally-invasive. The cargo of urinary-EVs therefore represents an attractive source for biomarkers to detect PCa. Recent developments in proteomics analysis allow deep profiling of clinical samples, including urinary-EVs.

Methods:
Urinary-EVs were isolated by ultracentrifugation from 3 control, 3 intermediate and 3 advanced PCa patients. Protein profiles in 1D-gel-separated fractions (5 bands/sample) were measured by label-free LC-MS/MS-based proteomics by spectral counting on a QExactive-platform. Protein identification and quantification was performed using MaxQuant, data analysis using the beta-binomial test, FunRich, Cytoskape, R2-software and transmembrane proteins were predicted using TMHMM-database.

Results:
In total, 3950 proteins were identified in urinary-EVs. This reached depth outperformed previously reported datasets. Cluster analysis clearly separated advanced from intermediate/controls. Differential proteins of intermediate PCa were associated to migration and transport, and profiles of advanced PCa were associated with metabolism, cell growth and transport. For metastatic PCa, epithelial to mesenchymal transition (EMT) is frequently observed. 311 candidate EV-proteins for advanced PCa (>1,5 fold change, $P<0.05$) highly correlated to the expression of 11 EMT related-genes in PCa-tissues of the Jenkins-dataset (GSE46691), in contrast to the intermediate PCa corresponding 135 candidate proteins. This demonstrates that urinary-EVs are useful for an early and minimally-invasive read-out for PCa-stage. For ELISA-based EV-capture, transmembrane proteins are important. Predictive analysis of transmembrane proteins indicated that 17/135 proteins more abundant in urine-EVs of intermediate PCa and 16/ 311 more abundant in advanced PCa have a transmembrane protein. These proteins represent attractive new candidates for disease-stage specific EV-enrichment.

Conclusion:
The identified protein profiles provide novel insights that may ultimately translate to a minimally-invasive detection method for PCa.

Keywords:
Prostate cancer, Urinary extracellular vesicles, Proteomics, Biomarker

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Longitudinal and Network-Based Biomarker Models for the Prediction of Ovarian Cancer

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Background
Ovarian cancer (OC) has the highest mortality rate of all gynaecological cancers. Commonly, diagnosis occurs at late stage where 5 year survival is between 4-19%. A crucial step in improving outcome is to improve early detection. With this in mind, we undertook a serum biomarker discovery study that aimed to develop novel longitudinal models for predicting OC. Secondly, we developed a parenclitic network approach for generating predictive topologies.

Methods
Biomarker discovery used pre-diagnosis serum samples taken <14 or >36 months to diagnosis from 19 Type I OC/Borderline cases, 31 Type II OC cases and 30 control subjects, sourced from UKCTOCS. Samples were extensively fractionated and analysed by LC-MS/MS with mass tagging. Selected candidates, including reported markers, were assayed in all serial samples from the same individuals. Longitudinal data was transformed into single trend indices and markers combined by logistic regression. A novel network approach was also applied to multiplex proximity-extension assay data for these samples and biomarker models derived.

Results
748 proteins were identified and altered candidates selected. Two multi-marker, longitudinal models (CA125+CHI3L1 and CA125+HE4) had significantly improved sensitivity (92.9% and 82.1% at 95% specificity) over longitudinal CA125 alone (75% at 95% specificity), P<0.05. Furthermore, predictive values increased 18-24 months before diagnosis, suggesting the models may improve on lead time to diagnosis. Parenclitic networks were able to detect OC with a sensitivity of 90% and have highlighted differences in LYPD3, TGFα, S100A and CD70 in cases versus controls.

Conclusions
We have developed two new longitudinal models that improve upon lead time and sensitivity for detecting ovarian cancer versus single marker tests; validation of these is now underway. Parenclitic networks provide an opportunity to further enhance diagnostic performance as well as uncovering the underlying biology of cancer progression.

Keywords
Ovarian cancer; biomarkers; networks; longitudinal analysis; prediction

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Comprehensive Analysis of Sulfonated Cysteines in Human Peroxiredoxin 1 by Orbitrap Mass Spectrometry

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Peroxiredoxin 1 (Prx1) is an antioxidant protein that reduces peroxides through 2 indispensable cysteines: a peroxidatic cysteine (CP) for peroxide reduction and a resolving cysteine (CR) for CP regeneration. CP reduction of peroxides leads to its oxidation, eventually to sulfonation and the inactivation of Prx1. Mass spectrometry has been an invaluable tool for the idenfication of sulfonated CP, but not CR. A recent study indicates that CR may also be sulfonated; still, the prevalency and function of CR sulfonation are unknown. In this study, we developed a sensitive method to comprehensively analyze sulfonated cysteines in Prx1 by optimizing both CID and HCD methods on the Orbitrap Velos MS. We discovered that CR sulfonation is indeed a reproducible post-translational modification that may play an important role in battling against oxidative stress in cells.

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Quantitative phosphoproteomics reveals T cell signaling drives HIV-1 spread

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HIV-1 exploits host cell factors and immune cell interactions to replicate and cause AIDS. To provide novel molecular insight, we developed a new approach to analyze dynamic signaling in mixed-cell populations. We temporally mapped cellular responses during HIV-1 spread between infected and uninfected T cells using quantitative phosphoproteomics. The identification of 28,853 phosphopeptides across four time-points revealed HIV-1 modulates >200 cellular proteins and numerous host cell programs were modified, many uncharacterized in HIV-1 infection. Strikingly, we found that HIV-1 spread at T cell-T cell contacts strongly activated T cell receptor (TCR) and co-stimulatory signaling. Functional studies showed the TCR and downstream kinases drive HIV-1 dissemination. Manipulation of signaling at immune cell contacts by HIV-1 defines a new paradigm of antigen independent activation of T cell signaling that HIV-1 uses to optimally replicate in immune cells.

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A compendium of co-regulated protein complexes in breast cancer reveals collateral loss events

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Background:

Protein complexes are responsible for the bulk of activities within the cell, but how their behavior and composition varies across tumors remains poorly understood. Recent large-scale efforts have characterized the proteomes of scores of tumor samples, permitting, for the first time, a large-scale assessment of the behavior of protein complexes across individual tumors and between different tumor types.

Methods:

We developed a data-driven approach that integrates tumor proteomic profiles with a large-scale protein-protein interaction network in order to identify protein complexes coherently regulated across tumor proteomes.

Results:

We identified a high-confidence set of 258 protein complexes coherently regulated across breast tumour proteomes. We identified complexes that are under- or over-expressed in specific breast cancer subtypes, and reproduced these associations in an additional patient cohort. By integrating genotype information we found that mutation or deletion of one subunit of a complex was often associated with a collateral reduction in protein expression of additional complex members. This collateral loss phenomenon was evident from proteomic, but not transcriptomic, profiles suggesting post-transcriptional control. Mutation of the tumor suppressor E-cadherin (CDH1) was associated with a collateral loss of expression of members of the adherens junction complex, an effect we validated through proteomic profiling of an engineered model of E-cadherin loss.

Conclusions:

We have developed a broadly applicable approach for discovering co-regulated protein complexes from proteomic profiles. We demonstrated the utility of this approach by identifying protein complexes highly co-regulated in breast cancer and found differential expression of specific complexes in different breast cancer subtypes. We observed a ‘collateral loss’ phenomenon that suggests that the recurrent mutations and deletions seen in cancer may impact not just the expression of individual tumour suppressor proteins but also entire complexes.

Keywords :

proteogenomics; cancer; data-integration; protein complexes; breast cancer

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Proteomic identification of elevated serum haptoglobin levels in dystrophinopathy

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Background:
Duchenne muscular dystrophy is a multi-system disorder associated with progressive skeletal muscle degeneration, cardiomyopathy and in a subset of patients, cognitive impairment. Our previous comparative proteomic analyses of tissue obtained from the mdx-4cv dystrophic mouse model have identified protein biomarkers of myofibrosis in cardiac and skeletal muscle and of reactive gliosis in the dystrophin-deficient brain. In order to correlate the muscle pathology to potential alterations in the circulatory system, serum from the mdx-4cv mouse has been studied by comparative proteomic profiling.

Methods:
Since one of the major bio-analytical challenges with the proteomic profiling of biofluids is the large dynamic range of protein expression, serum samples obtained from six-month-old wild-type and age-matched mdx-4cv mice were immunodepleted to remove the highly abundant proteins albumin and immunoglobulin. Label-free liquid chromatography mass spectrometry was subsequently used to identify differential protein abundance. Potential circulatory biomarkers of interest were further independently evaluated by a combination of comparative immunoblotting and enzyme-linked immunosorbent assays.

Results:
Proteomic profiling of serum revealed alterations in the abundance of 461 proteins; 360 of which were elevated and 101 of which were reduced. This proteomic survey resulted in the identification of a number of traditional serum markers of muscle pathology, including creatine kinase, carbonic anhydrase and lactate dehydrogenase. Of particular interest however, was the 50.3-fold increase in the abundance of the inflammation-inducible plasma marker haptoglobin. Its elevated levels were further confirmed by immunoblotting and enzyme-linked immunosorbent assays in serum obtained from both six-month- and twelve-month-old mdx-4cv mice.

Conclusion:
Augmented levels of haptoglobin are indicative of sterile inflammation in the dystrophic mouse model. Since its increased abundance in the circulatory system was detected by three independent methods, haptoglobin may represent a novel prognostic and/or therapy-monitoring biomarker to evaluate the inflammatory response in Duchenne muscular dystrophy.

Keywords:
Muscular dystrophy; circulatory biomarkers; haptoglobin; sterile inflammation

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Unbiased search of antibiotic targets by thermal proteome profiling

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Background
The rise of antibiotic resistance is a major threat for global human health, which is aggravated by the limited number of currently explored targets. Here, we use thermal proteome profiling (TPP) in bacteria to probe for potential new targets. TPP is based on the principle that proteins become more resistant to heat-induced unfolding when complexed with a ligand. By using a proteome-wide approach, this allows for the unbiased search of direct targets and off-targets of drugs, as well as their indirect downstream effects on biochemical pathways (Savistki, Science, 2014; Franken, Nat Protoc, 2015).

Methods
Two-dimensional thermal proteome profiling (2D-TPP) was performed in bacteria by incubating the cells with a range of compound concentrations and subsequently heating them to a range of temperatures (Becher, Nat Chem Biol, 2016). After cell lysis, the remaining soluble proteins in each condition were isolated and digested with trypsin. Peptides from each condition were labelled with tandem mass tags (TMT10) and analyzed on a Q-Exactive mass spectrometer.

Results
We obtained the melting profile of the proteome (meltome) of Escherichia coli. Bacteria were heated as whole cells, enabling the assessment of proteome wide thermal stability in vivo. Next, we performed 2D-TPP with currently approved drugs. We were able to correctly identify the molecular targets of these drugs.

Conclusions
We show that TPP can be used for the identification of antibiotic targets. This platform will allow for the discovery of potential new drug targets by screening molecules that impact bacterial cell growth.

Keywords: Thermal proteome profiling; Mass spectrometry; Drug discovery; Antibiotics; Bacteria

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Statistical analysis of ion intensities in label-free tandem mass spectrometry-based proteomics

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Background

The liquid chromatography tandem mass spectrometry platform (LC-MS/MS) is an indispensable tool for quantitative discovery proteomics. For spectral count data, which is based on the number of MS/MS spectra observed for a particular protein, we have developed the beta-binomial test (Pham et al., 2010; Pham & Jimenez, 2012). Recent reports have suggested that software tools developed for RNA-Seq data analysis such as edgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014) can also be employed. For ion intensity-based quantitation, we have recently developed a statistical model for technical variation that can be used in the generic s-test (Pham & Jimenez, 2016). However, the current implementation is hard to use for those who do not have access to the MATLAB platform. Hence, our aim is to provide a user-friendly R package and to compare the statistical test to other state-of-the-art methods, as well as to the spectral counting approach.

Methods

We downloaded a publicly available LC-MS/MS dataset that was recently used in a benchmark for quantitative proteomics (Choi et al., JPR 2017). The dataset contains 12 samples in which six proteins were spiked in at different concentrations. We processed the data using the MaxQuant software tool (Cox & Mann, 2008). The spectral counts and MaxQuant-normalized ion intensity values (MaxLFQ) were used for further statistical analysis.

Results

We obtained results from various statistical tests for both spectral counts and intensity-based quantitation. We compared the results and highlighted methodological similarities and differences of the methods. An appropriate technical variation model is crucial and can be easily embedded in the s-test. Finally, the s-test has been implemented in an R package called stest available at www.oncoproteomics.nl.

Conclusion

The R package stest is a useful contribution for statistical analysis of proteomics data.

Keywords

Statistical significance analysis, s-test, intensity-based quantitation, technical variation, LC-MS/MS

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PMD: A Resource for Archiving and Analyzing Protein Microarray data

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Background
Protein microarray is a powerful technology for both basic research and clinical study. However, because there is no database specifically tailored for protein microarray, the majority of the valuable original protein microarray data is still not publicly accessible.
Methods
To address this issue, we constructed Protein Microarray Database (PMD) based on MySQL and R, which is specifically designed for archiving and analyzing protein microarray data.
Results
In PMD, users can easily browse and search the entire database by experimental name, protein microarray type, and sample information. Additionally, PMD integrates several data analysis tools and provides an automated data analysis pipeline for users. With just one click, users can obtain a comprehensive analysis report for their protein microarray data. The report includes preliminary data analysis, such as data normalization, candidate identification, and an in-depth bioinformatics analysis of the candidates, which include functional annotation, pathway analysis, and protein-protein interaction network analysis. PMD (www.proteinmicroarray.cn) has collected 161 protein microarray data involved in 21 species and has over two thousand users from 20 countries.
Conclusions
With PMD, one can access all of the related information and the original protein microarray data in a “one-stop” fashion, with a capability of “one-click” data analysis. We strongly believe that PMD is a valuable resource for the research community by promoting protein microarray data sharing and facilitating data analysis.
Keywords
Protein microarray, Data archiving, Data analysis

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Analysis of an amyloid aggregation conformational conversion of TDP-43 in neurodegenerative diseases

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Background
TDP-43 proteinopathies (TAR DNA-binding protein) is a major deposited protein in amyotrophic lateral sclerosis and frontotemporal dementia with ubiquitin, which result in Alzheimer's and Parkinson's diseases. The evidence from pathological mutations identified that mutation are located in C-terminal peptide fragments in TDP-43. We presented the pathways of various TDP-43 mutated peptides using ion mobility-mass spectrometry in this case.

Method:
To characterize the impacts of mutations on the amyloid formation, including the wild type (D1), pathological mutations (G294V), sporadic mutations (G294A) and de novo single and triple mutants (GGG308PPP) were synthesized. After incubation in physiological condition (50mM ammonia acetate, pH=7, 37℃) for 10 days, all peptides showed pronounced oligomerization under the ESI-IM-MS (electrospray- ion mobility- mass spectrometry) conditions employed at Day 0.

Results:
For D1 peptide, the signals of larger oligomer, such as trimer and tetramer, are diminished significantly after 5 Days, suggesting elevated presence of amyloid fiber during this phase. On the other hand, pathological mutants (G294V and G294A) can be observed accelerated aggregation compared with D1 after 3th Day. Moreover, the replacement of proline in TDP-43 C fragment (GGG308PPP) is able to disrupt the aggregation characteristics and stabilize the oligmer structure with the signals maintenance after 10 Days. Our results suggest that GGG308PPP plays an important role in neurodegenerative diseases, and reduced the amyloid aggregation effectively.

Conclusions:
Through comparison of the D1, G294V and G294A are β-amyloid and accelerated aggregation propensity. Overall, the result demonstrated that GGG308PPP remained random-coiled and extremely low aggregation ability by ESI-IMS-MS analysis. This region may imply the importance in amyloidogenesis process.

Keyword:
neurodegenerative diseases, TDP-43(TAR DNA-binding protein), ion-mobility mass spectrometry (IM-MS)

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Structural Characterization of Glycoconjugates by Using Functional Carbon Materials with Hydrolysis Reaction in MALDI-MS

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Introduction

Due to its critical role in cell-cell recognition, signal transduction, a comprehensive structural anlalysis of glycan has been the focus of clinical biology in this decade. The functionality of glycan depends on their degree of isomeric diversity, which results from subtle changes in their sequential arrangements. Herein, we have demonstrated that functional carbon materials can be employed as the MALDI matrix and hydrolysis reagent to characterize the structure differences of saccharides without any chemical derivatization or using tandem mass spectrometry.

Methods

Functional carbon materials were developed through hard-template nano-casting, followed by post-chemical treatments. The carbon materials were mixed with different types of disaccharides. Then, the solution of the mixture was loaded on the MALDI-plate for the identification and structure analysis as described in the MALDI-TOF MS analysis.

Results

At 750 ppm concentration of carbon materials, the dissacharides sodium adducted molecular ion peaks, were predominantly present with relatively clean background. In addition, based on the properties of functional carbon materials, the proton transfer and hydrolysis reactions provide the specific fragments of glycan. Interestingly, we observed that m/z=201 fragments only occurred as a signature pattern to determine ß anomeric configuration. For the fingerprint fragments for the differentiation of six linkage disaccharides, the unique product ions served to distinguish linkages.

Conclusion

Based on these specific fragmentation patterns, we can easily deduce the structure of the carbohydrates by using carbon materials without tedious sample preparation. We intend to broaden our techniques for more complex and biologically relevant glycoconjugates.

Keywords: Glycoconjugates, Carbon materials. MALDI-MS

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Strategic Use of Targeted Proteomics for Triage of Biomarker Candidates

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Background: There is an urgent clinical need for identification and selection of biomarkers for early detection of aggressive cancers, including prostate, ovary and pancreas. Biomarker discovery studies frequently generate long lists of differentially abundant mRNAs and proteins derived from comparative studies of malignant and control tissues in relatively small numbers of samples. Translating these candidates based on differential expression into clinically useful assays requires validation at the protein level in accessible body fluids or archived specimens in an independent and appropriately sized population with adequate follow-up. Only a small percentage of candidates, often < 15%, survive even the first round of verification in independent sample sets.

Methods: In order to avoid the time and expense associated with the generation of antibody-based ELISA assays with sufficient specificity and sensitivity, we developed a series of mass spectrometry-based selected reaction monitoring (SRM) methods for targeted identification and quantification of candidate biomarkers, capable of simultaneously multiplexing 50 or more candidates with sub-ng/mL to 50 pg/mL detection limits.

Results: Two test panels were developed and tested: a panel of 61 candidate biomarkers for early detection of serous ovarian adenocarcinoma, and a panel of 52 candidate biomarkers for identifying aggressive as opposed to indolent prostate cancer from biopsy samples. The ovarian panel was tested in serum from 50 matched patients with benign serous gynecological lesions and 49 patients with high grade serous ovarian cancer; 8 of 61 candidates (13%) validated. The prostate cancer panel was tested on biopsies from Gleason 6-9 patients, 10 with no progression, 10 with biochemical recurrence, and 10 with metastatic progression; 5 of 52 (10%) candidates showed statistically significant association with progression.

Conclusions: Targeted SRM assays provide a viable strategy for rapid triage of candidate biomarkers prior to the development of ELISA-based assays.

Keywords: targeted proteomics; biomarkers; ovarian cancer; prostate cancer

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High-resolution quantitative proteomics applied to the discovery of biomarkers of innate immune response in tuberculosis.

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Background
Tuberculosis (TB) is caused by the bacterium Mycobacterium tuberculosis (MTB). Roughly one third of the world's population carries MTB in a dormant form. TB is responsible for the death of more than 1.8 million people each year. EMI-TB is an EU Horizon2020 funded action focused on selecting and developing a novel vaccine candidate for TB. One of the specific aims (partner CSIC, Vigo, Spain) is to establish a panel of protein biomarkers representative of those individuals that had been in contact with a patient with microbiological confirmed pulmonary TB but did not get infected. Those proteins could be indicators of a specific innate immune response.

Methods
Samples (serum, saliva and sputum) were collected from volunteer patients (TB: pulmonary TB; LTI: contacts with latent TB infection; non-LTI: contacts without evidence of latent TB infection). Quantitative proteomics were done using TMT10plex (Thermo) following manufacturer instructions. Peptides were fractioned by basic reversed phase chromatography. Fractions were analyzed in a LC-Orbitrap Elite. Raw data was processed using Proteome Discoverer 2.1 software. Modulated proteins were selected after exhaustive manual review of the processed data and non-parametric statistical analysis with R software.

Results
Most of the significantly modulated proteins when comparing non-infected vs. infected TB-contacts are found in saliva and sputum. Examples of those proteins include S100-A7, Ig-alpha-1/2 chain, Ig J chain, Small Proline-rich Protein 3, Cystatin-c, Basic Salivary Proteins 1,2 and 3, Mucin-7, Polymeric IG-receptor and Cysteine-rich Secretory Protein-3. Most of them are involved in defense against pathogens and innate immune response.

Conclusions
Results demonstrate that the saliva and sputum of non-infected TB-contacts pose a representative proteomic signature suggesting that innate immune response in both oral and nasal mucous membranes is a key event during the initial entry of the TB pathogen in the host.

Keywords
Biomarker, tuberculosis, vaccine, quantitative proteomics, innate immune response.

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Apolipoprotein E status determination in human saliva using LC-MRM: In Saliva Veritas?

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Background
Human apolipoprotein E (ApoE) is a 36 Kda glycoprotein implied in lipid transport in peripheral circulation and in the central nervous system. It exists three major isoforms, ApoE ε2, ApoE ε3 and ApoE ε4, differing by single amino acid substitutions involving cysteine−arginine replacements at positions 112 and 158. ApoE status is known to be a major genetic risk factor for late-onset Alzheimer's disease (AD), as well as, for cardiovascular diseases. In this study, we developed a specific salivary method for the ApoE phenotyping using non-invasive collection of saliva.
Methods
The set-up of the method has been performed using 102 human sera of Alzheimer patient containing all ApoE isoforms (Hirtz et al, 2016). The method was fully automatized (BRAVO AssayMap, Agilent T.) and adapted to saliva samples. Briefly, 150µL of neutral saliva were sampled (Salivabio swab), centrifuged 5' at 1500 G and transferred to 96-well plate. Proteins were denaturated with 50% trifluoroethanol, reduced (5 mM DTT), alkylated (20 mM IAA), and diluted to 10% trifluoroethanol. The samples were then digested using 2µg of trypsin 3 hours at 37 °C. Before analysis, samples were desalted using C18 tips, evaporated and resuspended in 20µL of A phase (3% acetonitrile, 0.1% formic acid) containing heavy internal standard peptides of ApoE ε2, ε3 and ε4 isoforms. Peptides were detected using LC-MRM.

Results
Our method represents a simple, non ivasive and efficient way to perform ApoE phenotyping using mass spectrometry. Our sample preparation has been optimized and automatized to facilitate the phenotype interpretation. Phenotyping was performed using a combination of the 4 tryptic peptides and validated using skyline software. Thanks to this approach, a high throughput screening of a population is highly feasible.

Conclusions
Our LC-MRM approach allowed to detect and quantify the 3 isoforms of the ApoE in human saliva.

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An unbiased protein association study on public human proteome reveals biological connections between co-occurring protein-pairs

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Background
Mass-spectrometry based proteomics produces large amounts of data. While typically acquired to answer specific biological questions, these data can also be reused in orthogonal ways to reveal biological knowledge. Here we present a novel method for such data re-use of public mass-spectrometry based proteomics data. Our method shows the biological importance of protein co-occurrence across all human proteomics data derived from PRIDE database

Method
Mass-spectrometry based proteomics data was obtained from the PRIDE. To calculate the weight of protein co-occurrence across experiments, we used statistical Jaccard similarity. Protein pairs with a similarity above 0.4 were mapped to knowledgebases; Reactome, Ensembl, IntAct, and CORUM, to assign potential biological relevance. Moreover, using published articles, we were able to determine possible biological connection between unannotated protein pairs.

Result
Of the 2325 protein pairs that pass the Jaccard similarity threshold, we have successfully been able to map 81% of pairs. 68% protein pairs were mapped with existing biological knowledgebases; Reactome, IntAct, CORUM, and Ensembl, and 13% with possible biological connection using Gene Ontology (GO) terms.
To verify the fundamental validity of our approach, we compared the level of biological association for the original results with that of protein pairs from randomized associations. When we mapped 2325 randomly selected pairs to the knowledgebases, we only found 40 annotated protein pairs (on average), and 69 annotated protein pairs (at maximum) over the 1000 iterations. As a result, the difference between real and random data is extremely significant.

Conclusion
We have successfully been able to map the vast majority of strongly co-occurring protein pairs with existing biological knowledge. Our approach shows that by re-using publically available data in a fully orthogonal way, effectively treating these data as a proteome-wide association study, we can extract various biologically meaningful patterns.

Keywords
protein-protein interaction, pathways, data analysis

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Proteomic Analysis of Signaling Specificity in Breast Cancer Cells

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Background
Receptor Tyrosine Kinases (RTKs)-dependent signaling control several cellular processes, including proliferation, migration and differentiation. The activation of specific signaling pathways depends on the engaged RTK ligand. However, the molecular bases of RTK-dependent signaling specificity are not still fully understood. As RTKs show aberrant signaling in many human diseases, i.e. breast cancer, studying RTK ligand-dependent signaling at a system-wide level is crucial to define the pathogenesis of RTKs-driven diseases.

Methods
The seven members of the Fibroblast Growth Factor Receptor (FGFR) family, which can be activated by more than twenty known Fibroblast Growth Factors (FGFs), represent a good model system to dissect signaling specificity in an unbiased manner. We used several breast cancer cell lines of distinct molecular subtypes stimulated with FGFs for different time points. We combined shot-gun mass spectrometry (MS)-based quantitative phosphoproteomics and proteomics (using the high-resolution QExactive HF instrument), bioinformatics, and functional assays to validate the results.

Results
Preliminary results of a label free phosphoproteomics experiment showed very good reproducibility. The stimulation of five different breast cancer cell lines with two FGFs binding to the same FGFR for different time intervals resulted in the modulation of signaling cascades in a stimulus-, time-, and cellular context-dependent manner. This finding was also confirmed at the proteomic level upon depletion of signaling adaptor proteins or kinases.

Conclusions
These findings suggest that the specificity of FGFR response in breast cancer, where FGFR signaling is often deregulated, depends on the engaged FGFR ligand and on signaling dynamics. Therefore, identifying signaling events associated to a specific ligand may have relevant pathological implications in breast cancer.

Keywords
RTK, cellular signaling, phosphoproteomics, quantitative proteomics, label-free proteomics, QExactive, Orbitrap

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Displacement chromatography in two-dimensional liquid–chromatography for proteomics

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Background:
Sample complexity of proteomes is a major challenge in bottom-up proteomics. The combination of cation-exchange chromatography (CEX) and reversed-phase (RP) chromatography represents a powerful approach to analyze complex proteolytic peptide mixtures by bottom-up proteomics. The displacement chromatography mode (DCM) promises an advantageous alternative to gradient chromatography mode (GCM) for separating proteolytic peptides by CEX. In this study, we developed an online 2D-LC-MSMS approach applying DCM in the first dimension (CEX) and compared the performance with that of an online 2D-LC-MSMS approach using GCM for CEX.
Methods:
5 µg tryptic peptides were injected into an online 2D-LC-MSMS system consisting of a capillary CEX column followed by a RP trapping column and a RP separation column. After sample loading by pulsed injections onto the CEX column, peptides were eluted from the CEX towards the RP trapping column by injections of increasing concentrations of ammonium acetate in step (GCM) or by pulsed injections of spermine (DCM). 2D-LC-MSMS data were processed and interpreted with OpenMS, MaxQuant and mathematica scripts.
Results:
The total amount of proteins and peptides identified by online 2D-LC-MSMS analysis of tryptic peptides in DCM was significantly higher and considerably more reproducible than in GCM. Elution in DCM provides a significantly better separation and a significantly higher identification rate of peptides with a net-charge state of +2, which represent the majority of peptides in mammalian proteomes after tryptic digestion. The better separation efficiency in DCM resulted in an identification rate increase of 159% for peptides with a net-charge state of +2.
Conclusions:
In comparison to gradient mode, the displacement mode for cation-exchange chromatography within an online 2D-LC-MSMS system for proteomics is significantly more efficient and capable to analyze sample amounts smaller than 10 µg.

Keywords
Proteomics; Displacement Chromatography; On-line Multidimensional Liquid Chromatography; Mass Spectrometry

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Annexin A3 as potential biomarker in first-onset psychosis

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The exact cellular and molecular mechanisms underlying onset and development of schizophrenia have not yet been completely elucidated, but the association of disturbed neuroplasticity and increased inflammation has gained particular relevance recently. The aim of this work was to identify in platelets biomarkers capable to distinguish non-affective psychosis, affective-psychosis and healthy control. We analyzed platelets from 11 non-affective psychotic patients, 8 affective psychotic patients and 16 healthy controls. Proteins were separated by bidimensional eletrophoresis, identified by LC-MS/MS and validated by western blotting. After identification of several proteins one of these aroused special interest: annexin A3. Annexins have already been described as responsible for phospholipase A2 activity modulation. In fact the role of annexin A3 inhibiting phospholipase A2 that is an enzyme associated with the membrane phospholipid metabolism. Increased PLA2 activity has been frequently reported in schizophrenia, whereas treatment with anti-psychotic drugs was found to reduce the enzyme activity to levels similar to those observed in control subjects. However the mechanisms underlying this reduction are not yet understood. Our results show that downregulation of annexin A3 is related to psychotic patients in general when compared to the control group. Consequently, it seems that is more related to the onset of psychotic symptoms than schizophrenia in general. We also observed a strong correlation of cytosolic calcium-dependent phospholipase A2 activity and annexin A3 that may be related to calcium dependence of both enzymes. On the other hand the strong but negative correlation between intracellular calcium-independent phospholipase A2 and annexin A3 may suggest a more selectivity inhibition of intracellular calcium-independent phospholipase A2 by annexin A3. Our findings seem to be promising in the search for a biomarker of FEP.

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The Power of Multiplexing- Combining TMT discovery and targeted label free quantitation for biomarker analysis

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Recently, isobaric labeling techniques TMT have become popular for biomarker discovery due to higher throughput and better precision and accuracy. The next verification step (10-50 patients) is still challenging when balancing the target numbers and devoted instrument time. Here, we propose a workflow for plasma proteomics from multiplexed TMT biomarker discovery to rapid and robust verification using capillary flow LC on a novel Orbitrap platform with up to 40Hz scan speed.
Plasma from normal and diabetic patients were depleted, digested, labeled with TMT six-plex reagents, mixed at 1:1 ratio, and fractionated. The fractions were separated in a 120min gradient followed by analysis on an Orbitrap instrument. Data were analyzed by Proteome Discoverer™2.2 software. For targeted analysis, the same depleted, but unlabeled, samples were separated at a flowrate of 2-5ul/min and analyzed using parallel reaction monitoring (PRM). The data were processed by Skyline or Spectronaut software.
The multiplexing capability of TMT labeling significantly saved instrument time and provided possibilities to perform extensive fractionation. Fractionation, combined with the new depletion columns, made the detection of plasma proteins spanning to 5 orders of magnitude accessible.
Over 200 peptide targets, which showed significant difference (>2 fold change) between normal and disease states in the above discovery experiment, were selected for label free targeted quantitation using PRM. Retention time prediction of unlabeled peptides was performed using adjusted hydrophobicity index calculations. LC separation at the capillary flow rate provided high sensitivity and offered improved retention time reproducibility and robustness. The fast scan speed on the new Orbitrap platform greatly facilitated the detection of hundreds of targets in a single experiment. This rapid and robust quantitation method confirmed the biomarker candidates found in isobaric labeled discovery experiment. The workflows described here enable the biomarker discovery and validation in a highly multiplexed and rapid manner.

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Proteomics Analysis of Single Cells Using a Carrier-assisted Targeted Mass Spectrometry Approach

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Background

Antibody-based flow cytometry and mass cytometry are the predominant technologies for proteomics analysis of single cells, however they share common shortcomings with other antibody-based methods and lack the ability for precise protein quantification. To tackle this issue, we developed a new antibody-independent, targeted mass spectrometry (MS) approach that couples carrier-assisted sample preparation to a highly sensitive targeted MS platform for enabling precise protein quantification in single cells.

Methods

We developed a preparation method that uses exogenous BSA protein as a carrier for lossless processing of single cells. A highly sensitive targeted MS platform, PRISM-SRM/PRM, was then used for quantification of key EGFR pathway proteins in 1-100 HMEC cells. Waters nanoACQUITY UPLC and Thermo Scientific TSQ Vantage or Q Exactive instrument were used for LC-SRM and LC-PRM analysis, respectively. Skyline was used for data analysis.

Results

As a proof-of-concept, 1-100 isolated HMEC cells were collected into tubes containing 50 µg BSA to prevent cell adhesion to the tube wall, and processed as easily as bulk cells with minimal sample loss. Consistent peptide recovery was observed across all the samples; multiple endogenous peptides were detected by PRISM-SRM at as low as 1-5 HMEC cells level, and the response was linear from 1 to 100 cells levels. Furthermore, the detection of SFADINLYR derived from NRAS in small numbers of HMEC cells (~80,000 NRAS molecules per HMEC cell from bulk cells) demonstrated that the carrier-assisted sample processing coupled to PRISM-SRM (cPRISM-SRM) can provide ~100 zmol level of sensitivity. Currently we are working on further improving the sensitivity, throughput and robustness for cPRISM-SRM.

Conclusions

cPRISM-SRM/PRM is a simple, effective targeted MS approach for sensitive and precise quantification of proteins in single cells. We envision that this new targeted MS approach will have broad utilities in biomedical research.

Keywords

Single cell; quantitation; carrier; PRISM-SRM

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Rapid Production of Virus Protein Microarray Using Protein Microarray Fabrication through Gene Synthesis (PAGES)

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Background:
Recently, more and more viral infectious diseases were emerging. As one of these viruses, Dengue virus was high mutable. Tens of million people were infected by Dengue virus every year. Up to now, besides that there was no effective vaccines and weak basic research, high effective platform tools were also short in the field of Dengue virus. At last, as a systematic platform, the conventional method for protein microarray was disadvantage in the long fabricated cycle, addiction to natural template and shortage flexible for high-mutable sequences.

Methods:
Consensus sequences of each serotype of Dengue virus were firstly calculated from more than 3000 published ployprotein amino acid sequences. Subsequently, all the consensus sequences were transferred into the corresponding gene sequences. After optimized, all the genes were artificially synthesized and the expression clones were constructed. Further, all the proteins were purified and characterized in a high-throughput way. At last, the protein microarray of Dengue virus could be fabricated and its applications were exploited.

Results:
Consensus protein sequences for all serotypes of Dengue virus were obtained. The corresponding genes were de novo synthesized and proteins were purified in high-throughput method. The Dengue virus proteome microarray with high quality was fabricated. The protein I-E and III-E were identified as the potential serum biomarkers and validated by ELISA. Similar dynamic IgG responses for I-E and III-E were observed.

Conclusions:
A new strategy PAGES, combined consensus sequence identification, gene synthesis and high-throughput protein purification, was developed. The Dengue virus protein microarray was fabricated in short time by exploiting this strategy. At last, the PAGES would be used for constructed protein microarray of other RNA viruses, because this procedure was commonality and no need to contact source of infection directly.

Keywords:
Protein microarray; Viral infectious diseases; Gene synthesis; Consensus sequence; Dengue virus

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A Human Lectin Microarray for Sperm Surface Glycosylation Analysis

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【Background】Glycosylation is one of the most abundant and functionally important protein post-translational modifications. As such, technology for efficient glycosylation analysis is in high demand. Lectin microarrays are a powerful tool for such investigations and have been successfully applied for a variety of glycol-biological studies. However, most of the current lectin microarrays are primarily constructed from plant lectins, which are not well suited for studies of human glycosylation because of the extreme complexity of human glycans.

【Methods】All of the lectins and lectin-like proteins were purified from yeast, and most showed binding to human glycans. To demonstrate the applicability of the human lectin microarray, human sperm were probed on the microarray. These bindings were validated by flow cytometry and fluorescence immunostaining. GST pull down and mass spectrometry analyzed which membrane proteins can bind with galectin-1.

【Results】Herein, we constructed a human lectin microarray with 60 human lectin and lectin-like proteins. Strong bindings were observed for several lectins with human sperm, including galectin-1, 7, 8, GalNAc-T6, and ERGIC-53 (LMAN1). Galectin-1 binds several membrane proteins including HSP90. Finally, functional assays showed that binding of galectin-8 could significantly enhance the acrosome reaction within human sperms.

【Conclusions】To our knowledge, this is the first construction of a human lectin microarray, and we anticipate it will find wide use for a range of human or mammalian studies, alone or in combination with plant lectin microarrays.

【Keywords】Glycosylation, Human lectin microarray, Human sperm

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Quality control of mass spectrometry proteomics data integration in neXtProt

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Background

As the reference knowledgebase for HPP, neXtProt integrates mass spectrometry (MS) data from proteomics experiments and upgrades the protein existence value to “evidence at protein level (PE1)” for entries matching the criteria agreed upon with HPP.

Methods

The latest PeptideAtlas Human and Phosphoproteome data, as well as manually curated data from MS experiments reported in the literature, are loaded in neXtProt. Spot checks have been carried out for all data sets to confirm the data are loaded, filtered, indexed, displayed and exported. More recently, global checks on the MS data integrated are carried out using SPARQL queries.

Results

Manual spot checks using random examples and special cases allow systematic errors to be quickly identified. For example, when a curated MS publication refers to a new type of PTM, we spot check that an entry retrieved by searching with this term contains the PTM and that it is found in the PEFF file for the entry. With the advent of the neXtProt RDF data model and SPARQL querying in 2015, global checks are now being carried out at each neXtProt HUPO reference release. We have thus tracked the evolution of (1) the number of MS peptides mapping to entries (quantitative metric), (2) the percentage of MS peptides mapping which are proteotypic (quality metric) and (3) the percentage of entries with a MS peptide mapping (coverage metric).

Conclusions

A combination of spot checks and global checks ensures the quality of the MS proteomics data integrated in neXtProt. Our metrics indicate a gradual increase in the quality of the MS data integrated in neXtProt.

Keywords

Data integration, proteomics, protein existence, validation, quality control

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Peptide-mediated ‘miniprep’ for high-throughput isolation of extracellular vesicles coupled to phosphoproteomics.

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Background

Extracellular vesicles (EVs) are cell-secreted membrane vesicles enclosed by a lipid bilayer that may be derived from endosomes or from the plasma membrane. Since EVs are released into body fluids, and their cargo includes tissue-specific and disease-related molecules, they represent a rich source for disease biomarkers. Here we explore the use of a, high-throughput EV capture method that we previously bench-marked by proteomics, for the analysis of phosphoproteins.

Methods

Extracellular vesicles were isolated from concentrated cancer cell line secretome using a peptide-mediated ‘miniprep’ isolation kit, the ME™ kit (New England Peptide). The peptide in the kit binds to canonical Heat Shock Proteins on the exterior of exosomes and EVs that subsequently can be precipitated using centrifugation. The extracellular vesicle pellets were lysed, digested and subjected to TiOx based stagetip phosphopeptide enrichment followed by subsequent label-free single shot LC-MS/MS (Q-Exactive) and data was searched using MaxQuant.

Results

Phosphoproteomics of EVs of the colorectal cancer cell lines HCT116 and HT29 yielded 11,185 phosphopeptides. These 11,185 phosphopeptides map back to 8052 phosphosites, derived from 2764 phosphorylated proteins including 163 phosphokinases. Current analyses focus on the glioblastoma cell lines U87 and EGFR mutant U87 .

Conclusions

Our data shows the feasibility of EV phosphoproteomics using a high-throughput, easy to use isolation method on cancer cell line secretome. We aim to further implement this method on different biological fluids, to enable development of non-invasive, liquid biopsy-based (predictive) diagnostics.

Keywords

Extracellular vesicles, phosphoproteomics, LC-MS/MS, ME™ kit, TiOx

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Mass-spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation

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Cellular heterogeneity is important to biological processes, including cancer and development. However, proteome heterogeneity is largely unexplored because of the limitations of existing methods for quantifying protein levels in single cells. To alleviate these limitations, we developed Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS), and validated its ability to identify distinct human cancer cell types based on their proteomes. We used SCoPE-MS to quantify over a thousand proteins in differentiating mouse embryonic stem (ES) cells. The single-cell proteomes enabled us to deconstruct cell populations and infer protein abundance relationships. Comparison between single-cell proteomes and transcriptomes indicated coordinated mRNA and protein covariation. Yet many genes exhibited functionally concerted and distinct regulatory patterns at the mRNA and the protein levels, suggesting that post-transcriptional regulatory mechanisms contribute to proteome remodeling during lineage specification, especially for developmental genes. SCoPE-MS is broadly applicable to measuring proteome configurations of single cells and linking them to functional phenotypes, such as cell type and differentiation potentials.

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Decoding the role of human cardiac stem cells in acute myocardial infarction using proteomic tools

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After an Acute Myocardial Infarction (AMI), Ischemia-Reperfusion (I/R) injury is characterized by a substantial decrease in the number of cardiomyocytes (CMs). Human myocardium harbors a population of endogenous cardiac stem cells (hCSCs) that is activated upon I/R injury, contributing to myocardial repair through the establishment of an auto/paracrine crosstalk between hCSCs and CMs in stress. Cardiomyocytes response to I/R has been extensively studied, while CSC role in myocardial I/R is still lacking characterization. In this study, we set up an in vitro human cellular model of myocardial I/R injury using donor derived hCSCs and CMs differentiated from human induced pluripotent stem cells (hiPSC-CMs) to further decipher the action mechanisms of hCSCs upon injury.
Monocultures and co-cultures of hCSCs and hiPSC-CMs were established. Ischemia was mimicked by culturing the cells at 0% O2 in Ischemia Mimetic Solution. In the reperfusion step, cells were placed back in their physiological conditions of oxygen (3%) and nutrients. The effect of I/R injury in hCSCs was assessed by total proteome analysis at different time points using nanoLC-MS (Eksigent LC4500 & TripleTOF 6600) and evaluated by IPA software. Growth factor secretion, cell viability, as well as hCSC proliferation were also evaluated.
Important features of I/R injury were successfully captured, namely CM viability loss, hCSC proliferation activation upon insult and the protective role of hCSCs on hiPSC-CMs. The culture readouts obtained together with the proteins identified in the different time points of the insult, allowed us to propose new possible players on hCSC regeneration response upon injury including activation of pathways related with cell proliferation, paracrine signaling, stress response and metabolism.

The human cellular model established in this work will allow further understanding on the molecular landscape of AMI, namely regarding hCSC regenerative response. This will potentiate the development of novel cell-and/or molecular-based therapies for myocardium regeneration.

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Post-translational modifications (PTMs) of huntingtin protein in Huntington's disease

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Background
Post-translational modifications (PTMs) of proteins regulate various cellular processes. PTMs of polyglutamine-expanded huntingtin protein, causative of Huntington's disease (HD), are likely modulators of HD pathogenesis. Previous studies have identified and characterized several PTMs on exogenously expressed huntingtin fragments, however none of these studies were designed to systematically characterize PTMs on the endogenous full-length huntingtin protein.
Methods
We found that full-length endogenous huntingtin, immunoprecipitated from HD knock-in mouse and human post mortem brain, is suitable for detection of PTMs by mass spectrometry. Label-free and Tandem Mass Tag (TMT)-based approaches were used to identify and quantify PTMs on the endogenous huntingtin protein.
Results
We identified around 3 dozens PTMs on the endogenous huntingtin, including 14 novel PTMs (6 serine and 1 threonine phosphorylation, and 7 lysine acetylation sites). Most PTMs were located in clusters, within predicted unstructured domains rather than within the predicted alpha-helical structured HEAT repeats. Employing quantitative mass spectrometry, we detected significant differences in the stoichiometry of several PTMs between HD and WT mouse brain. Mass spectrometry identification and quantitation were verified using phospho-specific antibodies for selected PTMs. To further validate our findings, we introduced individual PTM alterations within full-length huntingtin, and identified several PTMs that can modulate its subcellular localization in striatal cells.
Conclusions
These findings will be instrumental in the further assembling the huntingtin PTM framework, and validate PTMs of huntingtin as promising therapeutic targets for HD.
Key words
Huntington's disease; neurodegenerative disorder; post-translational modifications; mass spectrometry; TMT; human brain

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Cervical mucus proteome in endometriosis

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Endometriosis is a chronic gynecological inflammatory disease characterized by the presence of functional endometrial glands and stroma outside of the uterine cavity.The current gold standard for the diagnosis combines laparoscopic evaluation and biopsy of the visualized lesions. To date, several noninvasive biomarkers have been proposed; however, no definite diagnostic biomarker is yet available. The aim of this study was to characterize the CM proteome in patients with endometriosis using high resolution mass spectrometry-based proteomics, implemented by bioinformatic tools for quantitative analysis, in order to investigate the pathophysiological mechanisms of endometriosis.
Cervical mucus samples were collected from patients affected by endometriosis and fertile controls. An aliquot of the soluble acidic fraction of each cervical mucus sample, corresponding to 0.5 mg of total protein, was left to digest with sequencing grade modified porcine trypsin. The peptides were analyzed by LC-MS/MS on a high resolution Orbitrap Elite mass spectrometer and data were evaluated using bioinformatic tools.
We aimed at the first total profiling of the cervical mucus proteome in endometriosis. From the list of identified proteins, we detected a number of differentially expressed proteins, including some functionally significant proteins. Six proteins were quantitatively increased in endometriosis, almost all being involved in the inflammatory pattern. Nine proteins were quantitatively reduced in endometriosis, including some proteins related with local innate immunity (CRISP-3 and Pglyrp1) and protection against oxidative stress (HSPB1). Fifteen proteins were not detected in endometriosis samples including certain proteins involved in antimicrobial activity (SLURP1 and KLK13) and related to seminal plasma liquefaction and male fertility (KLK13).
This is the first application of high resolution mass spectrometry-based proteomics aimed in detecting an array of proteins in CM to be proposed for the noninvasive diagnosis of endometriosis. This chronic disease presents in CM an inflammatory protein pattern.

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Proteomic Characterization of Microneedle-Extracted Human Dermal Interstitial Fluid

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Background

With the advent of wearable sensing technologies such as the Fitbit, there is growing interest in-real time monitoring of an individual's physiological status using non-invasive techniques. Microneedles have been proposed as a novel minimally invasive technique for sampling dermal interstitial fluid (ISF) for clinical monitoring and diagnosis, but little is known about its composition. ISF is thought to be very similar to blood plasma as it is comprised of constituents that are continuously exchanged across the walls of capillaries. Herein, we report a novel and simplistic method which uses microneedles to extract large quantities of dermal ISF which was characterized using shotgun proteomics.

Methods

A microneedle array was created by adapting ultra-fine nanopen needles with a 3D-printed array holder to pierce the epidermis no more than 1.5mm from three healthy human donors. Protein content of ISF was quantitatively compared to patient-matched serum and plasma by TMT-tagged multidimensional LC-MS/MS analysis.

Results

Using the fabricated microneedle arrays, we routinely collected ~20uL of ISF per array. In total, 407 proteins were quantified with at least one unique peptide and of those, 135 proteins were differently expressed at least two-fold. Collectively, these proteins tended to originate from the cytoplasm, membrane bound vesicles, and extracellular vesicular exosomes.

Conclusions

This is the first comprehensive proteomic analysis of pure dermal ISF collected using a non-invasive microneedle array. Proteomic analysis confirmed previously published work which indicates that ISF is highly similar to both plasma and serum. However, unlike blister-derived ISF, there were no proteins uniquely identified in ISF. This is likely because the microneedle extraction method avoids the trauma caused by the generation of blister which artificially introduces intracellular proteins. Taken together, ISF could serve as a non-invasive alternative for blood-derived fluids with potential for real-time monitoring applications.

Keywords

Interstitial Fluid, Microneedle, Proteomics, Clinical Diagnosis, Physiological Monitoring

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Proteomic characterization of new transcription factors associated with a more invasive phenotype in colorectal cancer

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Background
In this study, we aim to identify transcription factors (TFs) associated with the metastatic phenotype in colorectal cancer. For the enrichment of TFs, we used a concatenated tandem array of consensus transcription factor response elements (catTFRE) on nuclear extracts of the cell lines.
Methods
A catTFRE plasmid was obtained from Dr Qin1. Here, we used two different isogenic pairs of colorectal metastatic cells, KM12SM/KM12C and SW620/480. Proteins were pulled-down using biotinylated DNA sequences and streptavidin beads, analyzed by LC-MS and quantified with the MaxLFQ algorithm. Bioinformatics analysis was done with DAVID , STRING and TFcheckpoint. Functional studies were carried out with siRNA interference. To analyze their potential prognostic value we used the GSE17538 dataset from GEO, which contains mRNA expression profile from 232 patients with colorectal cancer combined with QPCR and immunohistochemistry analysis.
Results
We found 85 TFs experimentally verified among the KM12 deregulated proteins and 87 TFs for the SW cells. Protein alterations were validated using different orthogonal approaches. TFE3, FOSL2, MAFG, TCF4 and CEBPB mRNA levels were upregulated in KM12SM, while SFPQ, SRSF3 and YBX1 were found downregulated. In SW620 cells the number of coincidences was limited with some of them showing opposing trends to KM12 cells. TFE3, TCF4 and MAFG increase prometastatic properties of cancer cells. On the other hand, low expression of SFPQ, SRSF3 and YBX1 were associated with higher recurrence in CRC patients. Low SRSF3 expression showed association with shorter disease-free relapse and poor prognosis.
Conclusions
We have identified a novel set of TFs involved in colorectal cancer metastasis. SRSF3 seems to function as metastasis suppressor in colorectal cancer and showed a promising prognostic value.
Keywords: SRSF3, Transcription factors, prognosis, metastasis, colorectal cancer
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Systematic development of sandwich immunoassays for the analysis of the secretome in plasma.

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Background
Sandwich immunoassays are important analytic and diagnostic tools for basic and clinical research. To expand the applicability beyond the more commonly analysed proteins, and the use of this concept to enhance analytical possibilities, functional pairs of antibodies and high quality protein standards are required.

Methods
Using the resource of antibodies from the Human Protein Atlas (HPA), full length proteins established by the Wallenberg Centre for Protein Research (WCPR) and plasma samples, we conducted a pilot study and screened 375 HPA antibodies for suitable pairs against 110 WCPR proteins. To achieve this, we developed a workflow based on multiplexed bead based protein capture and assessed suitable pairs for one biotinylated detection antibody at a time.

Results
Evaluating more than 1,100 expected pairs in 273,000 data points from in dilution series of both EDTA plasma and protein, we found 55 pairs (56%) for the protein standard only, while 24 pairs (25%) were also functional in plasma.

Conclusions
In summary, we have used a multiplexed immunoassay platform and resources of affinity reagents and secretome protein standards to screen for sandwich pairs that are now being applied for the discovery and validation of secreted protein candidates in plasma. In a next step, this approach can be used to quantify levels of secretome proteins in plasma sets of different disease cohorts. Thereby the most differential proteins can be selected, which ultimately reveals the levels of specific proteins being associated to definite diseases.

Keywords
Secretome, plasma, sandwich assays, assay development

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Plasma Proteomic Analysis of Intravenous Omega-3 Fatty Acid and Gemcitabine in Advanced Pancreatic Adenocarcinoma

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Background:Pancreatic cancer carries the poorest prognosis of all solid organ tumours. Administration of intravenous omega-3 fatty acid (n-3FA) in advanced pancreatic adenocarcinoma patients receiving gemcitabine chemotherapy in our institute shows disease stabilisation and improved progression free survival. Uncovering the underlying biological mechanisms that are responsible for these clinical effects will be investigated using high definition plasma proteomics.
Methods:Plasma from ten patients with histologically confirmed un-resectable pancreatic adenocarcinoma, collected after one month treatment with intravenous gemcitabine and n-3FA (treatment group, n=5) and intravenous gemcitabine only (control group, n=5). Plasma was 99% immuno-depleted using Seppro IgY14 + Supermix columns, reduced, alkylated and tryptically digested. A two-arm (treatment vs control group) experimental design where each individual sample was labelled with TMT-6plex (TMT:127-131), with a Quality Control sample comprising pooled samples from all ten patients labelled with TMT-126 for comparison. Combined TMT-labelled samples underwent high-pH reversed-phase fractionation. Fractions were injected into a QExactive-Orbitrap LC-MS/MS in triplicate and analysed on Proteome Discoverer 2.1 and Scaffold 4.7 (FDR 1%). Bioinformatic analysis was performed on Protein Centre for Gene Ontology Biological Process (GO-BP) enrichment analysis ($p<0.05$, Bonferroni corrected), Cytoscape for visualisation and KEGG pathway analysis.
Results:3476 proteins were identified. 125 proteins were significant markers of pancreatic cancer, including REG1A, LVVE and TFF. Anti-inflammatory markers (CRP, Haptoglobin and Serum amyloid A1) were reduced in the treatment group validating the anti-inflammatory effects of n-3FA. GO-BP enrichment analysis showed angiogenesis downregulation, complement immune systems upregulation and epigenetic modifications on histones. KEGG pathway analysis identified direct action via the Pi3K-AKT pathway, with decreased HSP90 and increased inhibitory protein 14-3-3. Serum amyloid A1 was significantly reduced ($p<0.01$) as a potential biomarker of efficacy for n-3FA.
Conclusion:Administration of n-3FA has anti-inflammatory, anti-angiogenic and pro-apoptotic effects via direct mechanism on cancer signalling pathways in patients with advanced pancreatic adenocarcinoma.
Keywords:Omega-3 fatty acid, pancreatic adenocarcinoma

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Proteogenomic analysis of alternative splicing: Protein isoforms as biomarkers for early detection of colorectal cancer

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Background: Early detection of colorectal cancer (CRC) and its precursor lesions (adenomas) is crucial to decrease CRC mortality. The fecal immunochemical test (FIT) is a screening test detecting protein hemoglobin. However, FIT performance is suboptimal and must be improved. Adenoma-to-carcinoma progression is accompanied by alternative splicing resulting in expression of tumor-specific protein variants. We aim to identify proteins derived from alternatively spliced RNA which might serve as candidate biomarkers for early detection of CRC.
Methods: RNA and proteins were isolated from 18 healthy human colon tissues, 30 adenomas and 30 CRCs. RNA was isolated from organoids derived from 5 adenomas and 4 CRCs. Samples were analyzed by RNA-seq (Illumina) and in-depth tandem mass spectrometry (QExactive). Proteomics workflow included sample fractionation in 10 gel bands and peptide separation on 90 min nanoLC-MS/MS gradient. SPLICIFY, a proteogenomic data analysis pipeline for identification of differential protein isoforms, was applied.
Results: SPLICIFY identifies differential isoforms on RNA level, which are used to enrich the protein sequence database against which mass spectra are searched. Splice-specific peptides are extracted and quantified to confirm differential splicing on protein level. In the tissue dataset, comparative RNA splicing analysis between CRCs and controls and between CRCs and adenomas revealed over 1000 events. These include isoforms known to be involved in cancer, such as RAC1, and isoforms detected both in tissue and organoids, revealing novel candidate biomarkers. Proteomics analysis revealed over 9000 protein IDs, including approximately 150 splice variants with peptide confirmation.
Conclusions: We confirmed that adenoma-to-carcinoma progression is accompanied by aberrant splicing. Organoid analysis allowed identification of gene isoforms from epithelial origin. Tissue analysis yielded tumor-specific splice variants representing novel protein candidate biomarkers for early detection of CRC. Diagnostic performance of these protein isoforms will be validated in stool and FIT samples.
Keywords: colorectal cancer, proteogenomics, alternative splicing, biomarkers

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The differential plasma proteome of obese and overweight individuals undergoing nutritional weight loss and maintenance

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Background
Proteomes profiling from human plasma via high-throughput quantitative pipelines is key for clinical research and biomarker discovery. Over many years, multiple research groups have pursued the goal of measuring “complete proteomes” with MS (the “protein number race”). Much less attention has been given to the “sample number race” that can ultimately lead to sufficient statistical power and deliver robust and reproducible biological findings, by taking into account diversity and variability of populations.

Methods
Using a scalable, versatile and highly automated workflow to analyze proteins in human samples at clinically relevant scale and performance, we profiled 1000 plasma samples from the multi-centered human dietary intervention study “DiOGenes”, which focuses on how obesity can be prevented and treated through diet. We generated differential plasma proteome profiles to identify proteins associated with weight loss and maintenance and explore their relation to BMI, fat mass, insulin resistance and sensitivity. Relative protein quantification was obtained at baseline and after combined weight loss/maintenance phases using isobaric tagging-MS. A Welch t-test determined differential proteins during intervention. Protein relationships with clinical variables were explored using univariate linear models, considering center, gender and age as confounders.

Results
473 subjects were measured at baseline and at 34 weeks; 39 proteins were longitudinally differential. Proteins with higher changes were SHBG, adiponectin, CRP, calprotectin, SAA, and PRG4, whose association with obesity and weight loss is known. We also identified new putative biomarkers for weight loss and maintenance. Correlation between PRG4 and PRAP1 variation and Matsuda-IS increment was shown.

Conclusions
MS-based proteomic analysis of a large cohort of overweight/obese individuals concomitantly identified known and novel proteins associated with weight loss and maintenance.
To the best of our knowledge, this is one of the few examples of MS-based proteomics applied to such a large cohort in clinical research.

Keywords
Biomarker; MS; Large-scale study

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DCBLD2 is a novel biomarker of myxofibrosarcoma invasion identified by global protein expression profiling

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Background
Myxofibrosarcoma (MFS) is a mesenchymal malignancy characterized by frequent recurrence even after radical wide resection. To optimize therapy for MFS patients, we aimed to identify candidate tissue biomarkers of MFS invasion potential.

Methods
Invasion characteristics of MFS were evaluated by magnetic resonance imaging and protein expression profiling of primary tumor tissues performed using two-dimensional difference gel electrophoresis (2D-DIGE). Protein expression profiles were compared between invasive and non-invasive tumors surgically resected from 11 patients.

Results
Among the 3,453 protein spots observed, 59 demonstrated statistically significant difference in intensity (≥ 2 fold) between invasive and non-invasive tumors ($p < 0.01$ by Wilkoxon test), and were identified by mass spectrometry as 47 individual proteins. Among them, we further focused on discoidin, CUB and LCCL domain-containing protein 2 (DCBLD2), a receptor tyrosine kinase with aberrant expression in malignant tumors. Immunohistochemistry analysis of 21 additional MFS cases revealed that higher DCBLD2 expression was significantly associated with invasive properties of tumor cells. DCBLD2 sensitivity and specificity, and positive and negative predictive values for MFS invasion were 69.2%, 87.5%, 90%, and 63.6%, respectively. The expression level of DCBLD2 was consistent in different portions of tumor tissues.

Conclusions
DCBLD2 expression can be a useful biomarker to evaluate invasive properties of MFS. Further validation studies based on multi-institutional collaboration and comprehensive analysis of DCBLD2 biological functions in MFS are required to confirm its prognostic utility for clinical application.

Keywords
myxofibrosarcoma, invasion, biomarker, DCBLD2, two-dimensional difference gel electrophoresis

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Proteomic Analysis of Staphylococcus aureus Planktonic and Hydrated Biofilms using TMT-based Mass Spectrometry

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Background
Staphylococcus aureus and coagulase-negative staphylococci comprises approximately 65% of infections associated with medical devices and are well known for their biofilm formatting ability. Biofilm-related infections are extremely difficult to eradicate owing to their high tolerance to antibiotics and host immune defences. Currently, there is no efficient method for early biofilm detection. A better understanding to enable detection of biofilm specific proteins in vitro and in vivo can be achieved by studying planktonic and different growth phases of biofilms using a proteome analysis approach. Our goal was to construct a reference map of planktonic and biofilm associated proteins of S. aureus.
Methods
S. aureus reference strain (ATCC 25923) was used to grow 24 hour planktonic, 3 day, and 12 day hydrated biofilms. Bacteria were grown in tryptic soy broth (TSB) liquid medium. Planktonic growth was used late logarithmic bacteria and the Centres for Disease Control (CDC) biofilm reactor was used to grow 3 day, and 12 day hydrated biofilms, respectively. Extraction, fractionation and concentration were performed using lysis buffer, probe sonication, 10KDa MWCO followed by 3KDa MWCO, respectively. Tandem Mass Tag (TMT) mass spectrometry (MS) was performed according to the manufacturer instructions (Applied Biosystems). Protein identification and relative quantitation of protein levels were conducted using ProteinPilot software which is purpose built for TMT analysis (Applied Biosystems).

Results
The present study showed that a considerable proteomic difference exists among planktonic and biofilms from S. aureus. A comprehensive knowledge of planktonic and biofilm associated proteins produced by S. aureus will provide a basis for future studies on the development of vaccines and diagnostic biomarkers.

Conclusions
In this study, we constructed an initial reference map of planktonic and various growth phase of biofilm associated proteins which might be helpful to diagnose biofilm associated infections.

Keywords: S. aureus, Bacterial Biofilms, CDC Bioreactor, TMT, MS

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Increased Electronegativity of High density lipoproteins Impairs its Functional Properties and Increases Risk of CAD

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Uremia patients have impaired high-density lipoprotein (HDL) function and a high risk of coronary artery disease (CAD), but the mechanisms are not understood. We studied the effects of HDL electronegativity—increased in uremia patients—on HDL properties and examined whether increased HDL electronegativity was a risk factor for CAD. HDL from 60 uremia patients and 43 healthy controls was separated based on electronegativity into 5 subfractions (H1-H5) with increasing electronegativity through anion-exchange chromatography. The percentage of H5 HDL (H5%) was significantly higher in uremia patients than in controls ($p<0.001$). Lipoprotein content in H1-H5 was analyzed and compared by using gel electrophoresis, MALDI-TOF and nanoLC-MS/MS with data independent analysis(DIA) . Apolipoprotein (Apo) AI concentration was lower and apolipoprotein modifications were more prevalent in uremia HDL subfractions than in control HDL subfractions. A higher degree of carbamylation of ApoA1 and ApoCIII were found in uremia HDL subfractions compared to healthy controls. Using fluorescence-based assays, we showed that anti-oxidant activity, anti-apoptotic activity, and cholesterol efflux capability were reduced in HDL subfractions from uremia patients when compared with control HDL subfractions. Multiple logistic regression analysis showed that H5% was associated with CAD risk in uremia patients. In conclusion, increased HDL electronegativity in uremia patients is accompanied by compositional changes and impaired HDL function. Furthermore, increased H5% is associated with increased CAD risk in uremia patients.

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Proteomes of paired plasma and cerebrospinal fluid and their association with biomarkers of Alzheimer disease

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Background

Shotgun proteomics using liquid chromatography tandem mass spectrometry (LC-MS/MS) can extensively characterize human plasma and cerebrospinal fluid (CSF) proteomes. Nonetheless, association between proteins present in both CSF and plasma has hardly been reported. In this study, we measured paired plasma and CSF proteome profiles of elderly subjects with MS-based proteomics to study the relationships between these two proteomes and decipher their association with CSF markers of Alzheimer disease (AD).

Methods

Cross-sectional analysis was performed on 120 older community-dwelling adults with normal (n = 48) or impaired cognition (n = 72). We quantified 423 proteins in plasma and 791 proteins in CSF samples. The proteome overlap between plasma and CSF was composed of 256 proteins. Correlation analysis was performed with the Pearson's statistics and Bonferroni correction for multiple comparisons.

Results

We identified 28 proteins with associated quantitative values between peripheral circulation and CSF in the central nervous system (p-value < 0.05 with correlation coefficient $R > 0.3$). We investigated the associations of the quantified proteins in plasma and CSF with the CSF concentrations of the well-accepted AD biomarkers Aβ1–42, tau, and hyperphosphorylated tau (P-tau). While, none of the plasma proteins displayed a significant correlation with such biomarkers, we reported 50 CSF proteins correlated with CSF tau and 46 associated with CSF P-tau, the majority of which were brain-enriched proteins. Only 4 proteins in the CSF showed relationship with CSF Aβ1-42.

Conclusions

We provide here a first comprehensive correlations of CSF and plasma proteomes, between each other and with AD biomarkers, as an additional resource to biomarker development for neurological disorders.

Keywords

Alzheimer's disease; Biomarkers; Cerebrospinal fluid; Isobaric tagging; Mass spectrometry; Plasma

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Thermal Proteome Profiling for Drug Discovery

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Background
The thermal stability of proteins can be used to assess ligand binding in living cells. We have earlier generalized this concept by determining the thermal profiles of more than 7000 proteins in human cells by means of quantitative mass spectrometry using isobaric mass tags, TMT10. Monitoring the effects of small-molecule ligands on the profiles delineated more than 50 targets for the kinase inhibitor staurosporine. Unexpected novel targets were identified and it was shown that changes in pathway activity could be detected by monitoring protein stability. Recently we improved the thermal proteome profiling, TPP, technology which enabled identification of novel and unexpected targets of histone deacetylase inhibitors.
Methods
We further developed the TPP approach by multiplexing temperatures and drug concentrations using TMT10. The resulting methodology 2D TPP is more sensitive because the target proteins have to exhibit dose dependent behaviour in their thermal stability changes and because both untreated and treated conditions are compared in the same experiment.
Results
We used 2D TPP to profile a marketed histone deacetylase inhibitor and found an unexpected target that would not have been detectable with the previous version of the methodology. Results on target validation as well as discussion of potential side effects and repurposing strategies will be presented. Data elucidating the mechanism of inhibition will be discussed.
Conclusions
Improved thermal proteome profiling technology enables identification of an unexpected target of a marketed histone deacetylase drug.
Keywords
Thermal proteome profiling, HDAC, drug discovery

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Characterization and Identification of Dityrosine cross-linked peptides using Tandem Mass Spectrometry

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Background: The use of mass spectrometry coupled with chemical cross-linking of proteins has become a useful tool for proteins structure and interactions studies. Unlike structural analysis of proteins using chemical reagents specific for lysine or cysteine residues, identification of gas-phase fragmentation patterns of endogenous dityrosine cross-linked peptides have not been investigated. Dityrosine cross-linking in proteins and peptides are clinical markers of oxidative stress, aging and neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). Although a unique biomarker in myriads of diseases, there remains a long standing question about how to specifically identify the peptides and proteins cross-linked via dityrosine in biological samples using tandem mass spectrometry.

Methods: In this study we have investigated and characterized the fragmentation pattern of dityrosine cross-links of peptides in ESI tandem mass spectrometry (MS/MS), generated by various techniques such as CID, HCD, ETD and ECD.

Results: Herein, we report the detailed fragmentation pattern of synthetically prepared dityrosine cross-linked of Aβ(1-16) to establish the generic rules for the gas phase fragmentation pattern of dityrosine cross-linked peptides in ESI-MS/MS. We used these rules to develop a method for identifying the dityrosine cross-links that forms by enzymatic peroxidation of α-synuclein, Aβ and hemoglobin using bottom-up proteomic workflow. The ability to define and characterize protein dityrosine cross-links solves a long-standing gap in our knowledge about the chemical nature of dityrosine cross-link in these neurotoxic proteins and peptides. Isotope labeling of dityrosine cross-linked peptides described in this study helped to resolve whether it forms through intra- or intermolecular mechanism. This technique is crucial for structural assessment of proteins undergoing oxidative stress.

Conclusions: Together these tools open up the potential for automated analysis of this naturally occurring post-translation modification in neurodegeneration as well as in other pathological conditions.

Keywords: Oxidative Stress, Dityrosine, Mass Spectrometry, Proteomics, Neurodegeneration

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The loss-less and nano-flow SPIDER fractionator for high sensitivity, high coverage proteomics

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Sample preparation workflows are a crucial part of mass spectrometry (MS)-based proteomics measurements. To achieve near-comprehensive identification and quantification of cellular proteomes, the combination of a first HPLC-based peptide fractionation orthogonal to the online LC-MS/MS step has proven to be particularly powerful. Here, were describe a novel approach termed “SPIDER fractionator”, in which the post-column flow of a nanobore chromatography system enters an eight-port flow-selector rotor valve. The valve then switches the flow into different flow channels at constant time intervals, collecting the fractions into autosampler vials of the LC-MS/MS system.

We demonstrate excellent sensitivity of our SPIDER fractionator by decreasing sample amounts from 100 µg to the sub-µg range, without any losses attributable to the fractionation system and while quantifying close to 10,000 proteins. Furthermore, we applied our system to an automated and in-depth characterization of 12 different human cell lines with a median depth of 11,300 proteins. We now routinely apply the SPIDER fractionator to various body fluids such as plasma, urine or CSF, as well as to tissue samples such as brain, heart or liver in order to generate in-depth peptide libraries. Such libraries are then used to transfer the peptide sequence information from the library to the actual sample runs. Thus, we are now able to quantify more than 10,000 proteins in single-shot runs of mouse brain tissue in combination with SPIDER-based peptide prefractionation.

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Proteomics of rare genetic diseases: impact of cystinosin mutations on protein stability and protein network

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Background: Cystinosis is a rare autosomal recessive storage disorder characterized by defective lysosomal efflux of cystine due to mutations in the CTNS gene encoding the lysosomal cystine transporter, cystinosin. Over 100 mutations have been reported, leading to varying disease severity, often in correlation with residual cystinosin activity as a transporter. However, the correlation between genotype and phenotype is not always clear and we applied different proteomics approaches to better understand the mechanisms of this disease. To unveil additional roles of cystinosin, we studied the protein interaction of the WT cystinosin and different mutations. Furthermore, we focused on an atypical mutation concerning protein glycolsilation, ΔITILELP, that sometimes leads to severe forms.
Methods: Co-immunoprecipitation and dynamic SILAC. All analysis were performed on a nanoRSLC Q-Exactive Plus MS.
Results: We found cystinosin interacts with almost all components of vacuolar H(+)-ATPase and the Ragulator complex and with the small GTPases Ras-related GTP-binding protein A (RagA) and RagC. These interactions are lost when cystinosin carries severe loss-of-activity mutations. We also showed that wild-type cystinosin is very stable, while ΔITILELP is degraded three times more rapidly . We observed that in the lysosome, ΔITILELP is still capable of interacting with the V-ATPase complex and some members of the mTOR pathway, similar to the wild-type protein. Our interactomic and immunofluorescence studies showed that ΔITILELP is partially retained at the endoplasmic reticulum (ER).
Conclusion: Our results show a dual role for cystinosin as a cystine transporter and as a component of the mTORC1 pathway, and provide an explanation for the appearance of Fanconi syndrome in cystinosis. We also found that the high turnover of ΔITILELP, due to its immature glycosylation state in combination with low transport activity, might be responsible for the phenotype observed in some patients
Keywords:Dynamic SILAC, Turnover, interactome, cystinosis, cystinosin

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The “Quantify then Identify” pipeline: a promising tool to minimize unexploited spectral information

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Background

Isobaric tagging is a method of choice in mass spectrometry (MS)-based proteomics for comparing multiple conditions at a time and performing protein biomarker discovery studies in biological, pre-clinical and clinical research. Nonetheless, some limitations of this technique lie in the data-dependent acquisition mode used for MS data acquisition; and the proteomic-typical data processing sequence that first identifies proteins in individual samples and then quantifies between multiple samples only those already identified proteins, leaving a large number of spectra unexploited. By contrast, in untargeted metabolomics, relative quantification precedes the identification of the already differentially displayed features.

Methods

We developed a bioinformatic pipeline that optimizes the processing of mass spectral data obtained from isobaric tandem mass tag experiments. Our method focuses on the tandem mass spectral level by first quantifying and then identifying (QtI), while preserving unidentified spectra for further investigations. The overall pipeline uses machine learning algorithms to optimize pre-processing parameters; quantifies; aligns spectra prior to merging; selects them; trains/tests multi-classification models; and –using a new peptide-match rescue approach– identifies more peptides than the classical identification/quantification method.

Results

We show herein that, by exploiting all isobarically-quantified spectra from four independent Escherichia. coli, human plasma and cerebrospinal fluid (CSF) datasets, the developed QtI pipeline outperforms a standard data processing pipeline in terms of peptide/protein identification rate and significantly alleviates missing data. The number of unexploited tandem mass spectra was reduced by 40% to 77% according to the sample and dataset type. In particular, the proportion of concomitantly quantified, matched and assigned spectra increased by 64% for the CSF samples.

Conclusions

Our future developments aims at simplifying the utilization of the QtI pipeline and implementing tools to identify the quantified but still unassigned spectra.

Keywords

Algorithms; Bioinformatics; Biomarkers; Discovery; Isobaric tagging; Machine learning; Protein identification; Quantification; Tandem mass spectrometry; Tandem mass tag

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Phosphoproteomics of an AML cell line panel pinpoints hyperactive tyrosine kinases as targets for treatment.

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Background: Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell disorder, characterized by expansion of immature leukemic blasts in the bone marrow. In AML tyrosine kinases have been implicated in leukemogenesis, and they are associated with poor treatment outcome. However, targeted therapy using kinase inhibitors (KIs) has had limited success, and improvements are needed in patient selection. Here we present a single sample, phosphotyrosine based phosphoproteomics workflow to identify driver kinases in AML cell lines and successfully select KIs for treatment.

Methods: Phosphotyrosine (pY) immunoprecipitation was performed on 16 AML cell line lysates and AML blasts of 2 patients using a pY antibody. In both the cell lysate and pTyr IP fractions, phosphopeptides were measured using nanoLC-MS/MS and identified using MaxQuant. Evidence for kinase activity was acquired through phosphokinase- and phosphosubstrate based ranking analyses.

Results: A total 4853 phosphopeptides were identified across all AML cell lines, corresponding to 4229 phosphosites (3605 class I) on 2267 phosphoproteins. Ranking analyses were able to successfully pinpoint hyperactive kinases in 6 cell lines with a known kinase mutation. Additionally, we identified kinase drivers in 2 more cell lines, which were proven sensitive to targeted inhibition. Six cell lines without a clear driver showed high MAPK1/3 activation, corresponding to the presence of RAS mutations. Selection for AML signaling pathway components allowed for clustering of cell lines according to their drivers. Importantly, analysis of two AML patients with a FLT3 mutation showed high Flt3 phosphorylation and activity, in line with cell line results.

Conclusions: Our data show the potential of pY phosphoproteomics to identify key drivers in AML in cell lines and patient samples. The predictive value of the phosphoproteome profiles was confirmed using targeted treatment of cell lines with KIs which successfully inhibited cell growth.

Keywords: Phosphotyrosine; Phosphoproteomics; Leukemia; Kinase Inhibitors; Tyrosine Kinase

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Toxins of Clostridium difficile reorganize the proteome of target cells

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Clostridium difficile is an anaerobic spore-forming bacterium and the leading cause of hospital acquired infective diarrhea. The main pathogenicity factors in C. difficile infections (CDI) are clostridial glucosylating toxins TcdA and TcdB. Both inactivate small GTPases of the Rho/Ras family. The binary toxin (CDT) is only expressed in a few pathogenic strains and catalyzes actin ADP ribosylation. All toxins induce symptoms like diarrhea, inflammation and necrosis.
Proteome responses of C. difficile toxins were analyzed in cell culture and animal models to elucidate unknown functions of this pathogen and its toxins. Besides biochemical analyses many MS techniques e.g. shot-gun proteomics, MRM, were used.
Glucosylation pattern of small GTPases by TcdA and TcdB were investigated by a quantitative MRM method. TcdA targets RhoA/B/C, Rac, Cdc42, RhoG, and Rap1/2, (H/K/N)Ras, and R-Ras. In contrast, TcdB did not glucosylate members of the Ras family.
Comprehensive SILAC and label-free proteome analyses had been conducted to investigate effects of TcdA, TcdB, and CDT on the proteome of target cells. Toxin-responsive proteins belong to different functional classes such as cell-cell junction, cell proliferation, cytoskeleton organization, signaling, and cell death. In case of TcdA, all toxic effects depend on the glucosyltransferase activity. In contrast, catalytically inactive TcdB alters the proteome of target cells and induces early cell death.
In a phosphoproteome approach about 1,200 significantly altered phosphosites in proteins down-stream of small GTPases were identified that link inactivation of small GTPases with responsive cellular proteins.
C.difficile and its toxin alter many cellular processes. MS techniques were able to uncover new functions of this pathogen and its virulence factors and spread more light on the molecular processes of the host response to these toxins.

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Uptake and distribution of the antidiabetic drug metformin into the perfused rat liver by MALDI-imaging

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Background: Metformin is the first line drug for the treatment of type-2-diabetes (T2D) prescribed to more than 100 million people worldwide. Several molecular mechanisms have been invoked to explain its antidiabetic effect, but the question remains open as to which of these dominate at therapeutic concentration. Insight into these matters is hampered by the lack of methods to assess its uptake and spatial distribution: in this study we leveraged MALDI-MSI to determine metformin concentration and distribution in the perfused rat liver, an experimental model offering complete control over drug delivery.

Methods: Wistar rat livers were perfused with Hanks-balanced salt solution supplemented with relevant nutrients and metformin in concentrations ranging from 1 µM to 1 mM. The perfusion medium was collected to monitor the effect of metformin on the production of glucose. Fresh-frozen 12 µm rat liver sections were imaged in Reflectron-Positive, LIFT MS/MS and Single-Reaction-Monitoring (SRM) modes, on a Bruker® UltrafleXtreme MALDI-TOF/TOF. Glucose concentration was measured spectrophotometrically by coupled enzymatic assays.

Results: Metformin-treated liver sections showed a distinct peak at m/z 130.16, unambiguously assigned by LIFT MS/MS and SRM, utilizing identities of various drug fragments reported in the literature. Metformin was detected evenly distributed in parenchyma irrespective of medium concentration and length of perfusion, with measured sensitivity below its established active clinical concentration. Kinetic analysis indicated that uptake of metformin from the perfusion medium to the liver parenchyma occurred with an estimated Km and Vmax at the order of 0.3 mM and 0.2 micromol/(gxmin), respectively.

Conclusion: These results show that MALDI-MSI can be applied to determine the spatiotemporal tissue concentration and distribution of metformin in the clinically relevant µM concentration range in mammalian tissues. Our results highlight the usefulness of MALDI-MSI as a tool in helping to define the molecular mechanism(s) underlying the effects of metformin in T2D and cancer.

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P-Mart - Interactive Online Software to Analysis and Exploration from Global Proteomic Datasets

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Background
The ability of clinical proteomics to successfully identify new prognostics or diagnostic markers of disease not only requires the generation of high quality data, but robust and reproducible statistical analysis. In particular, to assure the most robust statistical results it is essential to process these large and complex datasets in a manner that accounts for appropriate data issues, such as missing values. P-Mart is a new interactive web-based software environment that enables biomedical and biological scientists to perform in-depth analyses of global proteomics data at the peak-intensity level.

Methods
P-Mart offers a series of statistical modules associated with quality assessment, peptide and protein statistics, protein quantification and exploratory data analyses. The functions to perform these tasks are developed in the R or Rccp programming language, Rserve (https://www.rforge.net/Rserve/) is used to communicate between R and the web-service and the interface is developed in Java and deployed via Azure cloud services. A subset of the R functions are currently available via GitHub (https://github.com/pmartR/) and the web-service can also be installed via Docker Hub (https://hub.docker.com/r/pnnl/pmart-web/). Deployed in this manner, adding additional functionality to P-Mart is straight-forward through a standardized pipeline.

Results
Currently, P-Mart offers access to multiple cancer proteomic datasets generated through the Clinical Proteomics Tumor Analysis Consortium (CPTAC) at the peptide, gene and protein levels. P-Mart also allows users to upload private data via three easy to format data files. Analyses are performed in P-Mart via customized workflows and interactive visualizations, and yield a final report and results files in .csv format. The report allows any analysis to be reproduced easily by attachment to a publication.

Conclusions
P-Mart is deployed using Azure technologies http://pmart.labworks.org/. P-Mart is funded through the National Cancer Institute’s Informatics Technology for Cancer Research (ITCR) program under grant U01-CA184738-01.

Keywords
Statistics, Bioinformatics, Software, Reproducibility, Biomarkers

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Development of mass spectrometric-based assays for in situ target identification of covalent inhibitors

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Background
Elucidating drug activity and target specificity is a critical step for any drug development program. Several methods have been developed successfully to identify on- and off-targets from complex cellular environment, including affinity-based chemoproteomics and CETSA-proteomics. Recently there are increasing interests in developing covalent inhibitors for various therapeutic targets. We aimed to build proteomic methods to quantitatively assess protein labeling by cysteine covalent inhibitors in a dose-dependent or compound-dependent manner.

Methods
A PRM-based approach for on-target labeling evaluation, as well as an iodoTMT-based multiplexed cysteine-profiling assay, are implemented to assess proteome-wide target specificity of cysteine covalent inhibitors. IodoTMT specifically labels free cysteines from whole cell extracts allowing subsequent digestion and enrichment of cysteine-containing peptides using anti-TMT antibodies for LC-MS/MS identification and synchronized precursor selection (SPS)-based MS3 quantitation.

Results
We applied these assays to investigate recently reported covalent inhibitor that targets oncogenic Kras G12C mutant. From our studies, we identified and quantified >10,000 unique cysteine sites. We confirmed that the compound specifically and potently labels G12C cysteine residue in cells, while the off-target profile is comparable to other marketed covalent inhibitors. Some off-target hits from cysteine profiling are possible source of cytotoxicity.

Conclusions
By profiling cellular hits using targeted approach and unbiased global profiling method, we established an in-house platform for covalent inhibitor characterization.

Keywords
KRas G12C; PRM; Cysteine profiling; iodoTMT;

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Plasma proteins associated with advanced liver fibrosis in patients with nonalcoholic fatty liver disease.

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Background
Nonalcoholic fatty liver disease (NAFLD) may progress from simple steatosis to nonalcoholic steatohepatitis that leads to liver scarring (fibrosis). Individuals with advanced liver fibrosis or cirrhosis are at risk of complications such as liver cancer and liver failure. The aim of this study was to compare plasma proteins in patients with early NAFLD (i.e. early F0-F1) versus significant/advanced NAFLD (i.e. advanced fibrosis; F2-F4) to determine whether candidate proteins could be used to guide liver fibrosis staging.
Methods
Patients with biopsy and/or transient elastrography-proven early NAFLD (N=10) and advanced NAFLD (N=9) were matched for BMI, age, and gender. Plasma proteins were digested with trypsin and data were acquired by nanoLC/MS/MS (Sciex 5600). Data were searched using MASCOT and protein level information analyzed by ScaffoldQ+ and Progenesis Qi to determine consensus protein differences. Progenesis and Scaffold analysis included 277 and 235 proteins, respectively, at a false discovery rate <1%.
Results
Mean liver stiffness was 5.8±1.7 kPa in those with early NAFLD compared with 19.7±9.8 kPa in advanced NAFLD. Five consensus proteins were differentially abundant between groups: complement C7, alpha-2-macroglobulin, and fibulin-1 were greater than 1.8-fold elevated in patients with advanced fibrosis (P<0.02), while complement C8 gamma chain and alpha-1-antichymotrypsin were reduced in patients with advanced fibrosis (P<0.02). Receiver operator characteristic curve analysis showed that complement C7 alone was the best classifier for advanced NAFLD (AUROC=1) and complement C7 protein intensity was positively correlated with liver stiffness (Pearson's r=0.74, P<0.001).
Conclusions
Complement C7 levels significantly correlate with liver stiffness, and elevated plasma complement C7 appears to be an excellent classifier of advanced NAFLD. Further studies will be needed in a larger validation cohort to confirm the utility of complement proteins as biomarkers of advanced NAFLD.

Keywords
nonalcoholic steatohepatitis, steatosis

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Proteomics identified predictive biomarkers for response to treatment with pazopanib in sarcoma

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Background
Sarcoma is a rare malignancy with an aggressive clinical course. Pazopanib has been approved for the treatment of sarcoma. However, as only a limited proportion of patients exhibit favorable response to treatment with pazopanib, predictive biomarkers of response to pazopanib are urgently needed in sarcoma.

Methods
To identify the predictive biomarkers for response to treatment with pazopanib in sarcoma, we investigated four layers of proteome. We examined a set of sarcoma cell lines which showed the different response to the treatments with pazopanib. We investigated, 1) the global protein expression level by mass spectrometry, 2) the expression of all tyrosine kinases (TKs) by antibody-based proteomics, and the function properties of TKs which over expressed in the pazopanib-resistant cells, 3) the activity of TKs using global in-vitro kinase assay system, and 4) the amount of secreted proteins by antibody-conjugated beads technology.

Results
Firstly, we found the global proteome signature for resistance to pazopanib, in which 13 out of 90 TKs were identified by mass spectrometry. Secondly, we identified that five TKs were associated with the resistance to pazopanib by antibody-based proteomics. The overexpressed tyrosine kinases didn’t always provide apparent advantages to sarcoma cells, because the gene-silencing affected the cell growth for a limited type of TKs. Thirdly, we found the amount of TKs didn’t always correlate with their activity level. Finally, we identified the unique proteins which were highly secreted from the resistance sarcoma cells. Those proteins are the candidates to predict and monitor the effects of treatment of pazopanib.

Conclusions
In this study, we approached to the predictive biomarkers for response to treatment with pazopanib in sarcoma. Our results suggested the possible utility of proteomics for the exploration of predictive biomarkers for resistance to tyrosine kinase inhibitor.

Keywords: cancer proteomics, tyrosine kinase inhibitor, pazopanib, predictive biomarker, sarcoma

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Response prediction for platinum-based treatment regimens in non-small cell lung cancer using a protein-based assay

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Background
The majority of patients with non-small cell lung cancer (NSCLC) are treated with platinum-based chemotherapies in a one-size-fits-all approach. However, a significant number of patients do not benefit in terms of survival due to acquired or intrinsic drug resistance. Pinpointing NSCLC patients who are more likely to derive clinical benefit from platinum-based chemotherapies using molecular markers will improve clinical outcome and reduce both toxicity and health care costs.

Methods
Using label-free GeLC-MS/MS-based proteomics, we profiled the proteomes of a panel of human NSCLC cell lines with varying IC50 values for cisplatin in order to identify markers of cisplatin sensitivity. Moreover, we are currently profiling archived tumor resection material of a large number of NSCLC patients (n≥100) who received adjuvant platinum-based chemotherapy, and subsequently aim to correlate protein profiles to clinical outcome in order to validate our cell line lysate candidates and develop a predictor for recurrence-free survival.

Results
In a pre-clinical biomarker discovery effort, the proteomes of a panel of human NSCLC cell lines with varying IC50-values for cisplatin were analysed by label-free proteomics based on GeLC-MS/MS and quantified by spectral counting. We demonstrate that the levels of 395 proteins (out of a total of 3672 identified protein IDs) were significantly altered between cisplatin-sensitive and -insensitive cell lines. Statistical analysis and functional data mining revealed known and novel processes implicated in cisplatin sensitivity and resistance. Preliminary immunohistochemical analysis on tumor resection material of NSCLC patients indicates an association of selected cisplatin response prediction candidates with progression-free survival.

Conclusions
Our proteomic analysis of a panel of NSCLC cell lines yielded a promising pre-clinical protein profile for cisplatin response prediction, which will now be further explored and validated in clinical cohorts of NSCLC patients.

Keywords: label-free GeLC-MS/MS, proteomics, non-small cell lung cancer (NSCLC), cisplatin, biomarkers

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Identification of a novel therapeutic target for tongue cancer by antibody-based proteomics

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Background
Inhibition of kinases has recently garnered great attention as molecular therapy for cancer. Antibody-based proteomics approaches have been applied to tissue microarrays (TMAs) for screening of novel therapeutic targets and predictive biomarkers of cancer. Using antibody-based proteomics, we screened the expression profile of a kinome and identified a kinase termed “Z-kinase” as a novel candidate therapeutic target for patients with stage I/II tongue cancer. Here, we identified that the Z-kinase expression ratio is closely associated with poor clinical outcome in the early stages of tongue cancer and tested the effects of Z-kinase expression on the proliferation of cultured cells.
Methods
Protein expression of the Z-kinase was immunohistochemically examined in surgical specimens from 41 patients with stage I/II tongue cancer. Survival curves were analyzed using the Kaplan-Meier method. We newly engineered tongue cancer cell lines that express Z-kinase and investigated the effect of forced Z-kinase expression on cell proliferation.
Results
Z-kinase expression was detected in 12.2% of patients with stage I/II tongue cancer, and it was associated with clinical findings of malignant aggressiveness. Patients with Z-kinase expression had significantly poorer outcomes of disease-free survival than patients without Z-kinase expression ($p < 0.05$, Log rank test). The tongue cancer cell lines in which Z-kinase was forcibly expressed by retrovirus vector showed significantly increased cell proliferation compared with control cells ($p < 0.01$, Student’s t-test).
Conclusions
Z-kinase is a potential novel molecular target for tongue cancer therapy.
Keywords
Molecular therapeutic target, tongue cancer, kinome

MRM based absolute quantification of myeloid derived growth factor

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Background: Paracrine factors released from bone marrow-derived inflammatory cells mediate tissue repair after myocardial infarction (MI). One of these proteins is myeloid-derived growth factor (MYDGF), which promotes cardiac myocyte survival and angiogenesis in the infarcted myocardium thus leading to improved functional recovery and survival after MI in mice (Korf-Klingebiel et al. & Wollert, Nat Med 2015). Here, we developed an assay to measure MYDGF in patients with acute MI and to start elucidating its potential role as a biomarker.
Methods: Blood samples were collected from healthy volunteers and patients with acute MI. Two proteotypic peptides within MYDGF were selected for MRM analysis. These peptides were synthesized as stable isotope-labeled standards (SIS) and spiked into the digested samples to allow an absolute quantification using MRM and triple quadrupol-MS analysis.
Results: A sensitive and quantitative assay for MYDGF could be established. The assay was validated in heparinized plasma and EDTA plasma in terms of its linearity, recovery, and intra- and inter-assay reproducibility. MYDGF concentrations could not reliably be measured in serum due to MYDGF release from blood cells during coagulation. MYDGF concentrations were measured in plasma samples from 120 apparently healthy individuals. MYDGF levels were inversely correlated with age. MYDGF concentrations were then measured serially in 60 patients with acute ST-segment elevation MI. MYDGF-concentrations were significantly elevated after MI compared with age-matched apparently healthy individuals.
Conclusion: We developed a quantitative MYDGF assay based on MRM mass spectrometry using two unique peptides, each with two precursor fragment transitions. The reproducibility, matrix- and storage-effects were examined in accordance with FDA guidelines for bioanalytical method validation. MYDGF plasma concentrations decline with age in apparently healthy individuals. Plasma levels of MYDGF are significantly elevated in patients with acute MI. Using this assay, the potential role of MYDGF as a plasma biomarker can now be further explored.

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Identification of signaling pathways involved in colorectal adenoma-to-carcinoma progression

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Background
Colorectal cancer (CRC) develops in a multi-step-process from normal epithelium, through a pre-malignant lesion (adenoma), into a malignant lesion (adenocarcinoma). A minority of about 5% of adenomas will ultimately progress into cancer. To improve clinical practice it is important to identify the subgroup of colorectal adenomas that is at risk of progressing to cancer.
By extensive genomic analysis we have shown that gain of chromosome arm 20q is associated with colorectal adenoma-to-carcinoma progression. In addition we identified AURKA and TPX2 as major drivers of this amplicon. Yet, the activation status of these genes during the colorectal carcinogenesis and the downstream signaling pathways affected with the activation of these genes are not fully known.
In order to better understand the biology of adenoma to carcinoma transition, we performed a comprehensive analysis of phosphoproteomes at different stages of colorectal carcinogenesis.
Methods
Phosphotyrosine containing peptides were immunoprecipitated from 5 mg of colorectal adenoma (n=81) and colorectal carcinoma tissues (n=50) using agarose bead-coupled phosphotyrosine antibody P-Tyr-1000. A Q Exactive HF mass spectrometer was used to perform NanoLC-MS/MS. Spectral counts of phosphoproteins and ion intensities of phosphopeptides were defined by MaxQuant for relative quantitation of protein phosphorylation.
Results
Phosphotyrosine-based phosphoproteomics of the 131 colorectal tissue samples yielded 6056 phosphopeptides, corresponding to 2745 unique phosphoproteins including 183 phosphokinases. Data analysis to identify regulated phosphorylation states and inference of kinase activity is ongoing and the results will be presented.
Conclusions
We uncovered signaling pathways in different stages of colorectal adenoma-to-carcinoma progression. The results will be used to identify kinase drivers of adenoma-to-carcinoma progression.

Keywords
phosphoproteomics, colorectal cancer, adenoma, progression

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Quantitative proteomics reveals a crucial role of the allergen context during activation of dendritic cells

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Background
Until now the question how certain proteins can induce allergic immune responses in some individuals whereas others evolve tolerance is not fully answered. Studies suggest that allergenicity is a process not only depending on properties of the allergen itself but also on adjuvant effects evoked by costimulatory molecules contained in the allergen-source.
Methods
To investigate the allergenicity of the major birch pollen allergen Bet v 1 and the impact of known adjuvants coming from pollen, such as lipopolysaccharide (LPS), we performed quantitative proteome analysis of stimulated monocyte-derived dendritic cells (moDCs). We treated cells with birch pollen extract (BPE), a recombinant variant of Bet v 1 or LPS followed by proteomic profiling by means of high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) on a hybrid quadrupole-Orbitrap mass spectrometer using isobaric tags for absolute and relative quantification (iTRAQ®).
Results
We quantified more than 1,800 protein groups out of which 131 proteins were significantly regulated by either LPS, BPE, or rBet v 1. Enrichment analysis showed that most regulated proteins are involved in cytokine signaling, lymphocyte costimulation and MHC class II receptor activity. Within the affected pathways, we revealed differences in the response to the used treatments. We found highly regulated proteins after treatment with BPE and LPS, whereas the cellular response to the recombinant allergen itself was limited. Significant differences in the regulation were e.g. observed for GIT2 and MX1, both participating in signal transduction and cytokine signaling.
Conclusions
According to our results we consider Bet v 1 alone not to be able to induce an allergic immune response. Furthermore we believe that LPS is not necessarily needed to induce an allergic response but other context-dependent factors derived from the pollen are essential in this process.
Keywords
Quantitative proteomics, dendritic cells, allergy, birch pollen extract, Bet v 1

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Co-exposure of silver nanoparticles and Cd2+ induce disruption of the metabolic reprogramming in HepG2 cells

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Many studies have reported on the deleterious effects and mechanism of silver nanoparticle (AgNP) toxicity in a variety of organisms, but the possible toxicological interactions of AgNP and ubiquitous environment contaminants, such as cadmium, remains poorly understood. In this study, biochemical assays and mass spectrometry-based proteomics were performed with HepG2 cells after the co-exposure to AgNP+Cd2+ to explore cellular and molecular effects induced by the combination of these contaminants. The results are compared to the lone exposure of Cd2+ and AgNP. Cell viability (trypan blue and LDH leakage) and energetic levels (ADP/ATP) were slightly decreased after 4 h exposure to individual and combined contaminants; however, these endpoints were substantially reduced after 24 h co-exposure to AgNP+Cd2+ compared to the control and individual exposures, which only led to minor changes. Evaluating the quantitative proteomics results of 4,500 proteins across the experimental conditions showed the same trend with few dereguated proteins (<1%) in all conditions after 4 h exposure to contaminants. After 24 h approximately 7% and 2% protein deregulation were observed in cells exposed to AgNP and Cd, respectively. Surprisingly, with the co-exposure experiment, 43% of the cell proteome was deregulated. The great number of deregulated proteins from the AgNP+Cd experiment was surprising, and demonstrate a profound impact on liver cancer cells that to our knowledge has not been reported before. Briefly, the toxicity induced by AgNP+Cd involved (1) inactivation of Nrf-2, which resulted in antioxidant defense and proteasome related proteins down-regulation, (2) disruption of the cancer metabolic reprograming affirmed by low ATP levels and (3) increased protein synthesis in order to reestablish homeostasis. Thus, the adaptation strategy was not able to restore ATP levels and to avoid cell death.

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The Signaling Pathways of Folliculin: a Tumor Suppressor in Birt-Hogg-Dubé Syndrome

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Background

Birt-Hogg-Dubé syndrome (BHD) is an autosomal dominant syndrome caused by a loss-of-function germline mutation of the Folliculin (FLCN) gene. Upon LOH or a second somatic mutation patients suffer from benign skin lesions, spontaneous pneumothoraces, lung cysts and, most importantly, bilateral kidney cancer. Until now the function and molecular pathways that are controlled by FLCN are insufficiently clear.
Using a proteomic approach we wish to elucidate FLCN's biological role and uncover how FLCN loss leads to kidney cancer.

Methods

As a first step we have performed label-free GeLC-MS/MS-based proteomics on a BHD tumor derived cell line UOK257 (FLCN-) and the FLCN restored cell line UOK257-2 (n=2 per condition). For future proteomic analyses we will use the CRISPR/Cas9 technology to develop a new unique BHD model system in vitro.

Results

Proteomic analyses of UOK257 vs. UOK257-2 cell line lysates identified 5112 proteins, of which 451 were significantly different. Using our stringent EdgeR filtering script, 94 proteins were selected to be the most differential expressed between UOK257 and UOK257-2 cells. GO analysis revealed involvement of multiple biological processes such as MAPK signaling, cytoskeleton organization, cell migration and metabolism.

Conclusions

Proteomics of the BHD cancer cell line system has shown wide impact of FLCN status on the proteome, with multiple biological processes affected including MAPK signaling, cytoskeleton organization, cell migration and metabolism. On-going analyses of our newly created isogenic FLCN -/- cell lines will reveal how FLCN influences gene and protein expression in an oncogenic model setting. Future studies will focus on functional and clinical validation of the most important proteins.

Keywords

Kidney, hereditary cancer syndrome, cellular signaling

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BioUb strategy: a key tool to study protein ubiquitination applied to investigate the Angelman Sydrome

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Angelman syndrome (AS) is an autism-like rare neurodevelopmental disease, with so far no cure, that is caused by mutations that render the UBE3A gene non-functional in the brain. This gene encodes for an E3 ubiquitin ligase that functions to conjugate ubiquitin moieties to a unique set of proteins. A long-standing challenge has been to elucidate the underlying molecular basis for AS that may contribute to the development of effective therapies. In this regard, identifying in vivo UBE3A substrates is a priority. Our research group developed an innovative technique, termed the bioUb strategy, which consists on tagging ubiquitin with a 15 aminoacid long biotin-accepting peptide that serves for isolating ubiquitinated proteins that are further detected by LC-MS/MS. Following this approach we have identified a number of putative in vivo Ube3a substrates in the fly brain and at present we are working with bioUb mice to identify UBE3A ubiquitination substrates in the mammalian brain. Bearing in mind that protein ubiquitination is modulated by the coordinated action of E3 ligases and counteracting deubiquitinases (DUB), we are also focused on identifying the DUBs that are responsible for deubiquitinating UBE3A substrates. It could be anticipated that blocking the action of those DUBs the impaired ubiquitination pattern of UBE3A substrates occurring in AS patients could be at least partially restored. Therefore, we have developed an additional assay to monitor the formation of ubiquitin conjugates on specific proteins that allows validating our bioUb-MS-derived results and also detecting the DUB(s) responsible for counteracting the action of UBE3A. Overall, the combination of animal models of AS, innovative strategies to enrich ubiquitinated proteins and mass spectrometry technology will greatly contribute to expand our current knowledge in the pathogenesis of AS, which is the initial step towards the design of successful therapies to ameliorate the symptoms of the disease.

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Multi-omics approach to discover hidden coding regions in so-called non-coding RNA.

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Background
In recent years, a growing interest is directed towards the detection of translation of small open reading frames (sORFs).

Methods
We have reanalyzed 34 public human ribosome profiling datasets with a special focus towards detection of translation products from sORFs (other species as mouse, fruitfly, rat ... ongoing). A subcategory of these sORFs is located in so-called non-coding RNA regions. We used our in-house developed PROTEOFORMER pipeline to predict open reading frames (Crappé et al., NAR, 2015), with small modifications towards detection of small ORFs. For rescanning of public MS data deposited in the PRIDE repository, we modified the ReSpin pipeline using tools as pride-asap, SearchGUI and PeptideShaker. Next to experimental prove of translation (synthesis), other metrics based on phylogenetic conservation (PhyloP) and the typical periodic signal of ribosome profiling data (ORFscore) were calculated to further corroborate coding potential.

Results
Based on ribosome profiling evidence, more that 100 sORFs were identified located in ncRNA, occurring in more than five different RIBO-seq datasets (examples: JPX, Malat1). Half of all identified small ORFs demonstrate (1) very nice in silico coding potential measures based on ORFscore and coverage; (2) good phylogenetic conservation (using PhyloP). The MS-data rescanning is currently ongoing, but also yields many promising hits. All information can be extracted from our public repository www.sorfs.org, using a BioMart query interface.

Conclusions
Many so-called non-coding RNAs actually house putatively coding sORFs as demonstrated by this very comprehensive study using RIBO-seq and MS datasets, questioning the 100AA artificial length threshold that was put forward for ORF detection. It goes without saying that these findings need to find their way into annotated protein repositories such as UniProt and neXtProt. Future functional analyses will unravel the molecular function of these small translation products.

Keywords
RIBO-seq, MS, bioinformatics, multi-omics, small open reading frame, sORF

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Development of Targeted Mass Spectrometry-based Assays for ERCC1-202/XPF as Potential Biomarkers in Lung Cancer

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Background: DNA excision repair protein ERCC1 and DNA repair endonuclease XPF are key players in the nucleotide excision repair pathway and are involved in the repair of DNA lesions caused by platinum-based chemotherapy drugs. Although ERCC1 is a potential predictive biomarker of platinum-based chemotherapy efficacy in non-small-cell lung cancer, the lack of an antibody that specifically recognizes the active isoform, ERCC1-202, hampers the development of valid clinical assays. It is of great interest to discriminate the four ERCC1 isoforms (201, 202, 203, 204) to selectively quantify ERCC1-202. In addition, since the ERCC1-202/XPF dimerization is required to repair the platinum-DNA damages, the quantification of XPF protein adds further confidence to the analysis.

Methods: We have developed targeted mass spectrometry-based assays to discriminate the four ERCC1 isoforms and quantify the ERCC1-202/XPF proteins. The ERCC1-202/XPF complex was enriched from A549 lung cancer cell lysates by immuno-affinity purification. After proteolysis, the selected proteotypic peptides (PTPs) for ERCC1 and XPF isoforms were measured by parallel reaction monitoring (PRM) using a quadrupole-orbitrap mass spectrometer. The quantification of both ERCC1 and XPF isoforms was performed using stable isotope labeled peptides.

Results: While ERCC1-202 isoform does not contain a unique sequence from the other isoforms, we showed that the simultaneous detection of three ERCC1 PTPs, named isoform discriminating peptides, represents a signature of ERCC1-202. Moreover, we included control peptides to avoid ERCC1 isoform misclassification. Our immuno-affinity results show that XPF maintains a stable complex with ERCC1-202 isoform in A549 cells and that the ERCC1-202/XPF protein levels are correlated.

Conclusions: The use of targeted mass spectrometry-based assays to detect and quantify ERCC1-202/XPF proteins represents a more selective approach compared to the conventional immunohistochemistry assays in clinical samples. Therefore, the strength of this study is to guide clinicians' therapeutic decisions towards patient-tailored treatment strategies.

Keywords: ERCC1/XPF; biomarkers; PRM; accurate quantification

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Plasma Proteome Profiling Disentangles Caloric Restriction and Bariatric Surgery Induced Weight Loss

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Background
The blood plasma proteome is a unique reflection of an individual´s physiology, serving to integrate genetic backgrounds with complex environmental factors into a coherent and dynamic phenotype. In the face of several challenges, Mass spectrometry (MS)-based proteomics is shaping up to be an ideal technology for the discovery and quantification of proteins in this clinically relevant biofluid. Our 'plasma proteome profiling' pipeline could previously identify less than 500 proteins in undepleted plasma within 45 minutes and has now been drastically improved. Here, we studied two different approaches in weight loss intervention – caloric restriction and Roux-en-Y gastric bypass surgery – to demonstrate that plasma proteomics is able to disentangle highly complex physiological changes.
Methods
Automated plasma proteome profiling pipeline.
Results
We investigated a caloric restriction study and a bariatric surgery study by analyzing 1294 and 888 plasma proteomes, respectively. The proteomic coverage (1,546 proteins) was deep enough to quantify nearly the entire lipid homeostasis system, including liver presented lipoprotein receptors and enzymes.
Roux-en-Y gastric bypass results in strong weight loss, but this interventions concurrently lead to desirable outcomes such as increased insulin sensitivity in type 2 diabetes patients, for which the mechanisms are still unclear.
Of 60 significantly changed proteins in the bariatric surgery study, 38 were co-regulated in the caloric restriction study. These proteins include inflammation factors and adipocyte secreted proteins. The 12 proteins which were only significantly regulated in the bariatric surgery study included the well-known insulin resistance marker adiponectin and several other functional candidates. Those which were regulated in an opposing manner consisted of 15 proteins, including several apolipoproteins. These proteins are possibly metabolic regulators that are activated by the Roux-en-Y gastric bypass.
Conclusions
Plasma proteome profiling is able to disentangle very complex physiological changes and find new biomarkers.
Keywords
Plasma, biomarker, diabetes, obesity, metabolic syndrome

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Proteomics of the intestinal mucosal-luminal interface identifies biomarkers to improve pediatric inflammatory bowel disease diagnosis

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Background:
Improved biomarkers are needed to decrease the number of unnecessary invasive endoscopies performed for inflammatory bowel disease (IBD) diagnosis. This need is greatest in children, where the most frequently used IBD biomarker (calprotectin) suffers from low specificity. Patients with ulcerative colitis (UC) must also undergo extent of disease assessment which contributes to the therapeutic intervention decision process. This aspect of diagnosis currently relies exclusively on endoscopy and imaging and could benefit from reliable biomarkers.
Methods:
Proteins were isolated from intestinal mucosal-luminal interface (MLI) aspirates from the ascending colon (AC) (n=57) and descending colon (DC) (n=36), collected during diagnostic colonoscopy from a treatment-naïve pediatric patient cohort. The intestinal MLI proteomes of 18 non-IBD and 42 IBD patients were analyzed by liquid-chromatography tandem mass spectrometry. Multivariate analysis and receiver operating characteristics curves were performed on proteomic data to generate a panel of proteins to distinguish IBD from non-IBD and a panel for pancolitis vs non-pancolitis (UC disease extent).
Results:
A panel of 4 proteins was capable of discriminating active IBD from non-IBD with a sensitivity of 0.954 (95% CI: 77.16-99.88) and 1.0 (95% CI: 82.35-100) for the AC and DC respectively and a specificity of 1.0 (AC 95% CI: 81.47-100; DC 95% CI: 69.15-100) for both the AC and DC. A separate panel of 4 proteins distinguished pancolitis from non-pancolitis in UC patients with a sensitivity (95% CI: 59.04-100) and specificity (95% CI: 71.51-100) of 1.0. Select candidate biomarkers were validated on a non-invasive biospecimen (stool) by immunoblotting or ELISA, demonstrating their potential application in the clinic.
Conclusions:
The findings of this study are the initial work in enabling for more accurate diagnosis of IBD and aide in answering the increasing need for sensitive and accurate IBD diagnostic biomarkers.

Keywords:
IBD, Biomarkers, Proteomics, Pediatrics, Treatment-naïve

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Profiling the phosphotyrosine interactome of receptor tyrosine kinases

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Background
Activation of receptor tyrosine kinases (RTKs) of human is known to induce phosphorylation of their cytoplasmic tyrosine residues, which leads to the recruitment of proteins containing SH2 or PTB domain and then triggers the downstream signaling pathways. Aberrant activation or mutation in RTKs have been linked to the disease development, thus it is crucial to understand cell signaling events both in health and diseases mediated by the phosphotyrosine residues.
Methods
Around 1150 phosphotyrosine peptides (wild-type and mutants), representing all existing intracellular tyrosine residues of 58 RTKs, were synthesized, coupled to sepharose beads and then used as baits to enrich interactors from cell lysates. Bound proteins were digested with trypsin and sequenced by nLC-MS/MS on an Orbitrap mass spectrometry (~3500 hours), followed by the protein identification and quantification in Maxquant.
Results
The profiling work provides considerable supplementary information to current RTKs interactome, especially for those less-well studied RTKs. Analysis of cell signaling pathway indicates the interactors containing SH2 or PTB domain not only get involved in well-known pathways but are also related to T cell receptor signaling events. Not surprisingly, mutated tyrosine residues also showed considerable capacities of recruiting multiple functional partners. For example, the protein STAT5B strongly binds to the mutated peptide of FLT3 (D600Y), demonstrating the potential ability of this tyrosine residue to propagate extracellular signals into nuclei directly via the JAK-STAT pathway. Besides, several proteins without SH2 or PTB domain were identified as novel binders to both wild-type and mutant peptides. Follow-up experiments are ongoing to validate these findings.
Conclusions
The global analysis of the proximal interactome of the wild-type and mutated RTK family provides a deep insight into the redundant and specific signaling pathways mediated by receptor activation.
Keywords
Receptor tyrosine kinase; protein-protein interaction; cell signaling; SH2 domain; PTB domain; post-translational modification

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Chemical and phosphoproteomics for mechanism of action analysis of AKT inhibitors in breast cancer

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Background

Breast cancer is one of the leading cancer diagnoses and causes of cancer deaths for females worldwide. AKT belongs to the serine/threonine AGC protein kinase family and is member of the frequently deregulated PAM (PI3K/AKT/mTOR) pathway in solid tumors including breast carcinomas. Due to its key role in the PAM pathway, AKT has been object of intensive drug discovery research for several years leading to various small molecule inhibitors currently investigated in clinical trials.

Methods

We analyzed the action of five AKT inhibitors (AZD5363, GSK2110183, GSK690693, Ipatasertib, MK-2206) on HER2-overexpressing BT-474 cells. The phosphoproteomic experiment was performed in four biological replicates and included phosphopeptide enrichment by Fe3+-IMAC in HPLC format, quantification via TMTsixplex labeling and LC-MS3 analysis on an Orbitrap Fusion. For detecting the target spectrum of the five inhibitors, Kinobead γ pulldowns were carried out which were followed by LC-MS/MS analysis on an Orbitrap Elite and label free quantification. MaxQuant was used for data analysis.

Results

We present a quantitative phosphoproteome profile of BT-474 breast cancer cells upon individual treatment with five AKT inhibitors. Approximately 10,000 phosphorylation sites were identified and quantified in at least three out of four biological replicates. The inhibitor-responsive AKT core pathway was determined by combining the phosphosites which are significantly regulated by all five inhibitors. Inhibitor-specific changes of the phosphoproteome were integrated with Kinobead target spectra of the inhibitors to resolve specific modes of action.

Conclusions

We are now able to display the inhibitor-responsive AKT core pathway which consists of both proteins with and proteins without previously known contributions to the PAM signaling pathway. Our results can be further utilized for targeted assays as well as for validation of responsive markers for inhibitor treatments.

Keywords

AKT inhibitor, breast cancer, phosphoproteome, AKT core pathway, off-target

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Multi-omics Analysis of Esophageal Adenocarcinoma: How Lipid Metabolism Affects Cancer Progression

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Background

The incidence of esophageal adenocarcinoma (EAC) has increased in recent years, whilst the 5-year survival rate remains low at ~15%. EAC is associated with altered lipid metabolism, with obesity as a major risk factor. Conversely, cholesterol-lowering statin drugs are protective and attenuate growth and malignant potential of EAC cells. MALDI and spectroscopy studies report changes in the lipid profiles of EAC tissue compared to healthy squamous epithelium. Lipids affect cancer behavior by storing energy as well as regulating cell survival and apoptosis. However, the specific lipid metabolism pathways involved in the progression from the pre-cancerous condition called Barrett's esophagus (BE) to EAC remain unclear.

Methods

To study these pathways, we conducted mass spectrometry (MS) -based proteomics and lipidomics experiments on 7 cell lines representing non-dysplastic BE, high-grade dysplastic BE and EAC.

Lipid and proteins were extracted from cell pellets using a biphasic TBME/MeOH method. Proteomic profiling was performed on a QE+ MS (Thermo). Untargeted discovery lipidomics experiments were performed on a 1290 Infinity II/6550 Q-TOF LC/MS system (Agilent) whilst targeted MRM experiments were performed on a 6490 Triple Quadrupole MS system. Combined analysis of the proteomic and lipidomic changes were performed to identify lipid metabolic pathways differentially expressed in EAC progression.

Results

Our results show that lipids involved in energy storage and regulation of cell survival are associated with disease progression. Furthermore, alterations in the fatty acid chain length and number of unsaturated bonds within multiple lipid classes were detected between the disease stages. Proteomics experiments revealed the enzymes which are likely to cause these alterations in lipid metabolism.

Conclusions

A combined proteomics and lipidomics mass spectrometry-based approach revealed alterations in lipid metabolism associated with the disease progression of BE to EAC. Future work will verify candidate molecules in patient samples.

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Selectivity determination of 1000 small molecule kinase inhibitors using chemical proteomics

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Background
Protein kinases have emerged as a leading class of drug targets in pharmaceutical research and are frequently addressed using small molecule inhibitors. So far, only a small fraction of the 518 human protein kinases have been targeted with small molecule inhibitors even though many kinases play an important role in human diseases. Using a library of 1,000 kinase inhibitors (clinical drugs and compounds from medicinal chemistry programs in the pharmaceutical industry), we aim to identify small molecules for the hitherto-untargeted kinome.

Methods
To profile the target space of all kinase inhibitors, we use the kinobead technology which features seven unselective small molecule kinase inhibitors immobilized on Sepharosebeads for affinity kinome enrichment from cell lysate. Pre-incubation of lysates with two inhibitor concentrations leads to a competition between the free inhibitor and kinobeads for the active site of the kinase and thus enables dose dependent target identification. In combination with quantitative mass spectrometry the chemical proteomics approach leads to the identification and quantification of over 300 kinases in a single experiment.

Results
Here we present the results of an initial screen of 1,000 small molecule kinase inhibitors and their target space using an optimized chemical proteomics workflow. This workflow includes on-bead digestion and a decreased amount of cell lysate so that sample preparation and measurement time is reduced by a factor of ten. Overall, we generated a large data matrix identifying the targeted kinome. The selectivity varies between the compounds ranking from 1 to over 100 targets per inhibitor.

Conclusions
This work highlights the ability of chemical proteomics to identify the target space and selectivity of small molecule kinase inhibitors. The results indicate that there already is chemical matter for the majority of the human kinome but a substantial part of the disease relevant kinome is still untargeted.

Keywords
Chemical proteomics

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Scanning the precursor range with OpenECHO: Adding precursor specificity back to DIA analysis

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Background: Data-independent acquisition (DIA) methods with large precursor-isolation windows (including SWATH-MS) suffer from loss of precursor information for the recorded fragment ions. Scanning quadrupole DIA methods, such as SONAR, address this lack of information by scanning the precursor m/z range in a continuous fashion, resulting in fragment ion signal present only in a specific subset of scans. Here we present OpenECHO, a novel software for targeted analysis of scanning DIA acquisition schemes.

Methods: Here, 1.5 ug of HeLa cell lysate was separated on a C18 analytical reverse phase column over 120 minutes and analyzed on a G2-XS QTOF mass spectrometer (Waters Corporation), operated in SONAR mode. The scanning quadrupole covered a range of 400 to 900 m/z, with an isolation width of 23 Da, a duty cycle of 1.0 second, and a spectral collection rate of 200 Hz.

Results: We developed OpenECHO, a novel open-source software capable of targeted analysis of scanning quadrupole DIA data. Using the scanning quadrupole dimension, OpenECHO can compute the likelihood for a fragment ion to have originated from a designated precursor ion by identifying the scans in which it uniquely occurs (e.g. identifying the ion as it enters and leaves the quadrupole window), thus increasing specificity and removing interference. OpenECHO extracted SONAR ion traces of 205 078 peptide precursors contained in the pan-human assay library from the HeLa data (Rosenberger et al, 2014). We quantified 2819 proteins (22 907 peptides) at 1% protein FDR in a single 90 min LC-MS/MS injection. Compared to SWATH-MS (Rosenberger et al, 2014), OpenECHO shows 30% increase in protein identifications using the exact same assay library; while OpenECHO increases protein identifications by 42% compared to an untargeted approach.

Conclusions: OpenECHO is the first open-source software for targeted analysis of scanning quadrupole data. It outperforms untargeted approaches and SWATH-MS.

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Protein biomarkers of metastatic propensity in prostate cancer

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Background:
In 2015 more than 8.8 million people died of cancer. The most prominent type of cancer in the male population is prostate cancer with an estimated 25.2% of newly diagnosed male cancer cases in 2016. Due to improved treatments and tests for early detection (i.e. PSA-Test), the overall survival rate is about 94%. Even though surgical treatments have a very high cure rate they also have side effects such as incontinence or erectile dysfunction. The tests for early detection are also by no means accurate as they can be influenced by other diseases and as such cause false-positive results. To minimize surgical interventions, more specific diagnostic biomarkers of prostate cancer metastatic propensity would better identify risk patients.
Methods:
Two prostate cancer cell lines, one with high metastatic potential (PC3) and one with low metastatic potential (DU145) were injected subcutaneously into SCID mice. The tumors were excised and prepared for proteolytic LC-MS-MS analysis. Briefly, tissues were lysed using a probe sonicator, DTT was used to reduce disulfide-bonds and IAA was added to tag cysteine. Trypsin was used as protease.
Results:
A total of 2361 proteins could be identified of which 1645 were quantified using DDA label-free quantification. After statistical analysis of the data (T-Test, PCA) 43 potential markers for highly metastatic prostate cancer and 34 potential marker for non-metastatic prostate cancer were identified. 6 Proteins of the coagulation pathway were found to be significantly regulated 2 of which, CD59 which inhibits the membrane attack complex and CD55 which inhibits the formation of C4b2a were up-regulated in highly metastatic cells.
Conclusion:
Mouse xenograft studies identified several proteins associated with metastatic phenotype. These will be studied in human prostate specimens of varying Gleason scores for validation. Secreted proteins will be examined in patient blood samples.
Keywords:
Cancer, Biomarker, Proteomics, Label-free Quantification

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Enrichment strategies for improvement of mass spec analysis of chemical cross-linked peptides.

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Background
Chemical cross-linking in combination with mass spectrometry is a powerful method to determine protein-protein interactions. However, this method suffers from low identification rates without enrichment/fractionation, as the typical yield of cross-linked peptides is less than 1 % of total identified peptides. In this study, we evaluated multiple, widely used enrichment/fractionation techniques and benchmarked newly developed SCX spin columns for cross-linked peptide analysis using an Orbitrap Fusion Lumos mass spectrometer.
Methods
Different amine-reactive, homobifuctional crosslinkers including DSS, DSSO and DSBU were used to crosslink protein and protein complex standards. Cross-linked samples were reduced, alkylated and digested with trypsin. Cross-linked peptides were pre-fractionated on SCX stage tips, SCX spin columns and SEC Superdex Peptide PC column (GE Health). Enriched samples were separated using a 50cm Thermo Scientific™ EASY-Spray™ column and an EASY-nLC™ 1200 UPLC system in 60min gradient, followed by detection on the Thermo Scientific™ Orbitrap™ Fusion Lumos™ mass spectrometer. Data were analyzed using Thermo Scientific™ Proteome Discoverer™2.2 software and XlinkX node.
Results
Selective fractionation of cross-linked peptides by SEC or SCX using an offline LC approach is widely used for improvement of interaction sites identification. However, this strategy requires special equipment or results in a large number of fractions. To overcome these limitations, we have designed and used new spin columns containing polymer-based strong cation exchange resin and used them for selective enrichment of cross-linked peptides. Our method was developed by optimizing enrichment efficiency with respect to pH and ionic strengths of the buffers/solutions used and benchmarked against well- established methods. For newly developed SCX spin columns, we identified a similar number of crosslinked peptides as by other traditional enrichment methods in only 2 fractions.
Conclusions
Overall, the combination of MS-cleavable crosslinking with MS3 acquisition methods and SCX pre-fractionation enables sensitive and selective analysis of protein-protein interactions in complex samples.

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Lyn, A Novel Putative Drug Target For Osteoporosis Management

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Background: Postmenopausal osteoporosis affects millions of women worldwide. Estrogen plays a key role in maintenance of bone mass, majorly by adversely affecting osteoclast (bone resorbing cells) differentiation and function. However, the precise molecular mechanism/s involved in this phenomenon is still an enigma. Unravelling mechanism/s of its action may aid in enhanced understanding of underlying pathophysiology of postmenopausal osteoporosis and identify novel therapeutic targets for its efficient management in future. We thus aimed to identify estrogen regulated proteins during different stages of osteoclastogenesis.
Method: Alterations in the proteome during different stages of human osteoclastogenesis on day 1, 5, 10 and 14 in response to 1nM 17β-Estradiol were discerned using 8plex-iTRAQ. Selected protein Lyn was validated by qPCR, immunoblot. Its functional significance in osteoclastogenesis was investigated by silencing studies.
Results: Through 8plex-iTRAQ technical replicates, 1,342 common proteins (≥2 unique peptides) were identified; of which, 489 proteins were differentially regulated (fold change ≥1.5) at atleast one stage of differentiation. Gene Ontology enrichment analysis of these proteins invoked our interest in Lyn, a tyrosine kinase, belonging to catalytic activity class. We observed Lyn to be upregulated in response to estrogen on day-1 of osteoclastogenesis. Lyn is reported to interfere with RANK-RANKL signalling, which is indispensable for osteoclastogenesis. Validation of Lyn transcript and protein expression in response to estrogen revealed it to be upregulated throughout osteoclastogenesis. Phosphorylation of tyrosine residue-Y507 (inhibitory motif) retains Lyn in a ‘closed’ (inactive) form. Our study showed estrogen significantly decreases Lyn phosphorylation (Y507) thereby releasing Lyn from its inactive conformation. Lyn silencing during human osteoclastogenesis showed significant reduction in inhibitory effect of estrogen on osteoclast differentiation and resorption potential.
Conclusion: We propose a novel mechanism by which estrogen negatively regulates osteoclastogenesis by triggering overexpression and increased activation of Lyn leading to decreased osteoclastogenesis.
Keywords: Osteoclastogenesis, Estrogen, 8plex-iTRAQ, Lyn

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Proteogenomics of ADAR-mediated RNA editing in Drosophila melanogaster

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Background
RNA editing is a type of posttranscriptional modification made by specific enzymes. Specifically, adenosine-to-inosine editing of RNA catalysed by ADAR enzymes is a most common type of editing in human and model animals and it is shown to modify also protein sequences. As far as we know, RNA editing products were not yet studied at the level of protein in the proteome-wide manner. We propose the proteogenomic approach for RNA editing sites search at the proteome level.

Methods
Three shotgun proteomic datasets of Drosophila melanogaster have been analyzed: two taken from publicly available repositories, and the brain proteome was obtained with Orbitrap LC-MS/MS. Customized proteomic databases were generated from the transcriptomic data taken from the open data and a RADAR database. These databases were used in combinations with UniProt database for protein identification. Two edited sites were selected for targeted analysis by MRM to observe the unedited-to-edited peptide concentration ratios in flies raised in different ambient temperatures.

Results
The total number of about 50 edited proteins was found in all datasets, 7 of them being shared between two of three proteomes studied. The edited proteins are attributed to synapse signalling, cytoskeleton components and RNA helicase activity. Two edited sites in syntaxin 1A and complexin belonging to a presynaptic vesicle machinery were selected for validation by targeted analysis. The results obtained for two selected sites have shown remarkably constant ratios of unedited-to-edited protein variants in flies raised under different ambient temperatures. This ratio was low in syntaxin 1A protein (2-5% of edited form), but much higher in complexin (about 30%).

Conclusions
The work demonstrates the feasibility to identify RNA editing events at the proteome level using proteogenomic approach. Targeted proteomics may be used to monitor the state of edited proteins in different conditions.

Keywords
Proteogenomics, RNA editing, Drosophila melanogaster, neuroproteomics.

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Comparison and optimization of global pSTY and pY-specific enrichment methods for mass spectrometry-based phosphoproteomics

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Background
Protein (de)phosphorylation is a key step in cellular signaling . For comprehensive profiling by tandem mass spectrometry, phosphopeptide enrichment is required. Over the past decade phosphopeptide enrichment methods including TiO2, IMAC and anti-pY antibodies have been reported and improved. Here we present a comparison and optimization of global pSTY and pY-specific enrichment methods.
Methods
Global pSTY enrichment methods were compared in single-shot label-free LC-MS/MS: (1) TiO2 beads using lactic acid or TFA to reduce acidic peptide binding. (2) Subsequently, we compared the optimized TiO2 method with a new magnetic bead IMAC approach (CST). (3) For pY-specific enrichment the effect of detergents on background peptide capture was assessed. (4) Additionally, the batch-wise pY enrichment method will be compared to a packed-tip format to reduce a-specific binding.
Results
(1) Lactic acid reduces acidic, non-phosphopeptide binding to TiO2 beads: 89% phosphopeptides vs 78% for TFA. However the overall phosphopeptide yield was higher for TFA: 7484 phosphopeptide IDs for TFA vs. 6053 for lactic acid per enrichment at 500 µg peptide input.
(2) IMAC showed superior selectivity for phosphopeptides compared to TiO2: 94% vs 78% phosphopeptides per enrichment. Moreover the overall yield was also higher for IMAC: 7752 phosphopeptides compared to 7529 for TiO2.
(3) 1% of the neutral detergents lauryl maltoside and octyl glucoside in the IP and wash buffers did not change the yield of pY phosphopeptides compared to control (no detergent). The typical phosphopeptide enrichment was 5% with an average yield of 541 phosphopeptides at 5 mg peptide input.
Conclusions
Although TFA improved the phosphopeptide yield in TiO2-based enrichment compared to lactic acid, IMAC proved to be the best performing method with respect to yield and selectivity. Background binding of non-phosphorylated peptides in pY IP remains an unsolved issue. Detergents do not improve selectivity for phosphopeptides.
Keywords
Phosphoproteomics, TiO2, IMAC

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Proteomic analysis of extracellular vesicles from clinically available volumes of CSF: application to Alzheimer's disease

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Background: Cerebrospinal fluid (CSF) contains extracellular vesicles (EVs) with undisclosed biomarker potential for neurodegenerative diseases. CSF EVs have been studied mainly using chromatography and ultracentrifugation methods. However, these techniques do not allow the establishment of a high-throughput proteomic method to analyze samples from clinically available volumes of CSF (0.5-1 mL). Here we explored the use of a peptide affinity method (Vn96 peptide) to isolate an EV-enriched fraction from CSF and we used this new approach to identify potential Alzheimer's disease (AD) biomarkers in CSF EVs.
Methods: CSF samples were obtained from the biobank of the University of Perugia. EV isolation was carried out on 1 mL of CSF using the peptide-affinity method that precipitates EVs based on binding to heat shock proteins on the vesicle surface. High-resolution mass spectrometry was used to characterize the proteome of CSF EVs, while functional annotations were investigated using enrichment analysis.
Results: In total, 936 proteins were consistently identified in all the CSF EV samples obtained via the peptide-affinity method. More than 60 frequently identified exosomal proteins were found in CSF EVs, including proteins involved in exosomes biogenesis, heat shock proteins, and tetraspanins. Substantial overlap (63%) was found between the CSF EV proteome isolated by the new method in comparison to gold-standard ultracentrifugation.
When applied to pooled CSF from AD and control subjects we found 39 protein up-regulated in AD EVs, while 25 were down regulated compared to CTRL EVs. Up-regulated proteins in AD EVs were related to extracellular matrix organization and cell adhesion, while down-regulated protein were associated with immune response and B-cell signaling.
Conclusions: The peptide-affinity method allows for the isolation of EVs from small volumes of CSF and is suitable for the analysis of CSF EV proteomes from patients with neurodegenerative diseases.
Keywords: extracellular vesicles, mass spectrometry, cerebrospinal fluid, biomarker, Alzheimer's disease

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Data independent versus data dependent acquisition mass spectrometry for proteomic classification of colorectal cancer subtypes

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Background: Colorectal cancer (CRC) is a heterogeneous disease, molecular subtyping may help to predict drug response and outcomes. Recently, a classification based on 4 consensus molecular subtypes (CMS) was proposed. Here we compared data dependent (DDA) and data independent (DIA) acquisition methods for distinguishing the four CMS subtypes, with the aim to build a proteomic classifier for CRC.
Methods: CRC tissues from the 4 CMS subtypes were lysed, digested and desalted. Four pools (CMS1,2,3,4) were constructed, including 10 patients per group. Single shot runs in triplicate were performed for both DDA and DIA using a 120 min gradient on a QExactive HF. For DDA, a top-15 method was used, while DIA data were acquired using variable acquisition windows of 20, 40 and 60 Da. DDA data were searched using the MaxQuant computational platform; while DIA data were searched using the Spectronaut software against a spectral library built with 12 DDA runs of the pools, including 3697 protein groups and 29793 peptides.
Results: About 3600 protein groups were identified in DIA mode, showing an overlap of 90% with DDA analysis.
DIA showed a high level of data completeness, with an average number of missing values per sample of 2% (DDA=19%). Technical variation was also lower with DIA, with a median CV of 5%, vs 16% of DDA analysis. DIA analysis was able to detect 68% of the candidates differentiating the 4 CMS subtypes found with DDA. CMS1 subtype specific markers were associated with complement activation and immune processes, CMS2 with mitochondrial organization and oxidative metabolism, CMS3 with glucose metabolism and vesicle transport; while CMS4 with extracellular matrix and epithelial-mesenchymal transition.
Conclusions: DIA is a robust method for biomarker discovery in clinical settings, the high reproducibility makes it amenable for CRC proteomic subtyping.
Keywords: DIA, DDA, mass spectrometry, colorectal cancer, subtype, CMS

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Identification of candidate serum biomarkers of pediatric Growth Hormone deficiency using SWATH-MS and feature selection

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Background
There is no single biochemical parameter available for the accurate diagnosis of growth hormone (GH) deficiency in children. There is a need for non-invasive biomarkers.
Methods
Serum samples from 15 GH deficiency patients and 15 healthy controls were depleted from the seven highest abundance proteins (Hu7 MARS kit, Agilent) and 50 ug of protein were digested with trypsin. An ad-hoc spectral library was built from the samples using LC-MS/MS top65 DDA runs with a 2 hour 5% to 30% ACN gradient in a nanoHPLC nLC415 (Eksigent) coupled to a Q-TOF mass spectrometer (Sciex TripleTOF 5600+). Samples were then run using a variable SWATH method using the same LC-MS/MS platform, and the quantitative data for each protein in the library were extracted using the MS/MSALL with SWATH Acquisition MicroApp v2.0 (Sciex).
For selecting those proteins that best discriminate between individuals with GH deficiency and controls, a feature selection workflow including three different algorithms for classification (RF-Boruta, SCAD-SVM, PAM) was applied using the bootfs R package. In order to obtain a final biomarker set with the minimum number of features able to obtain the best classifying performance, ROC analyses were performed on the top-ranked proteins and their combinations. The significantly affected pathways and gene ontology over-represented components were analysed using iPathwayGuide (Advaita)
Results
263 proteins could be confidently detected and quantified on each sample. The top 10 biomarker candidates are FIBA, APOA4, FHR4, SAA2, FINC, CXCL7, C4BPA, APOC4, F13A and APOF. The combination of three proteins, APOA4, FHR4 and CXCL7, showed the best classification performance for our data.
Conclusions
The analysis of serum proteins by a SWATH approach is a useful method for discovering potential biomarkers of GH deficiency in children. Three proteins classified GH deficiency patients and controls with best performance.
Keywords
Growth hormone deficiency; SWATH; feature selection; biomarkers

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Comprehensive high-throughput analysis of IgA1 O-glycoforms by a sequential deglycosylation protocol

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Background
IgA1 with galactose (Gal)-deficient hinge-region (HR) O-glycans (Gd-IgA1) plays a key role in IgA nephropathy (IgAN). The serum level of Gd-IgA1 is elevated in the majority of IgAN patients. To characterize the involvement of IgA1 in the development and progression of IgAN, O-glycan heterogeneity and attachment sites of Gal-deficient O-glycan should be analyzed, as each HR has nine potential sites for O-glycosylation.
Methods
The analysis of IgA1 O-glycan heterogeneity involved two steps. First, performing IgA1 HR O-glycopeptide high-resolution liquid chromatography-mass spectrometry (LC-MS) profiling enabled the identification of IgA1 O-glycoforms, defining macroheterogeneity of IgA1 O-glycans. Second, using electron-transfer dissociation (ETD) tandem MS enabled the characterization of individual IgA1 O-glycofoms in terms of the sites of attachment of the O-glycan chain(s), defining microheterogeneity. To increase the throughput of the analysis, we developed in-house automated program named Glycan Analyzer to identify and quantify the O-glycoforms. Further, we developed a sequential enzymatic deglycosylation protocol, which leaves only Gal-deficient O-glycans on HR; this workflow was tested to analyze the O-glycosylation of serum IgA1 from a patient with IgAN.
Results
After neuraminidase treatment, 13 glycopeptides corresponding to HR variants with 3-6 O-glycans attached to the His208-Arg245 amino-acid backbone were detected quantitatively by Glycan Analyzer. After sequential enzymatic treatment, only Gal-deficient O-glycans remained on HR glycopeptide; the sites with Gal-deficient O-glycans, including structural isomers, were successfully identified by using on-line LC ETD tandem MS. The results of our study show that Gal-deficient O-glycans are attached at specific sites. Once the attachment sites of Gal-deficient O-glycans are localized by ETD tandem MS, comparison of the isomeric distributions can be easily accomplished in a large number of samples.
Conclusions
Our new high-throughput workflow is a promising powerful method to identify specific glycoforms and specific sites with Gal-deficient O-glycans in IgA1 from patients with IgAN.

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Phosphoproteomics in metastatic colorectal cancer for predicting response to anti-EGFR therapy

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Background: The discovery of the key role of the epidermal growth factor receptor (EGFR) and its downstream signalling effectors in the pathophysiology of colorectal cancer (CRC) has resulted in the clinical use of targeted therapies in the treatment of metastatic colorectal cancer (mCRC). However, clinical benefit to EGFR blockade is observed in only a subgroup of mCRC patients wild type for KRAS, NRAS and BRAF. In this study, we performed phosphoproteomics of mCRC patient-derived xenograft models to shed new insights into cellular signalling of mCRC, identification of alternative drug targets and predictive markers.

Methods: Pharmacologically (cetuximab response) and genomically characterised mCRC-PDX models (1,2) were analysed (n = 41). Phosphopeptides were enriched by (p)-tyrosine immunoprecipitation and titanium dioxide. Dedicated bioinformatics was used to identify hyperactive kinases and predictive biomarkers for response to cetuximab. Identified alternative drug targets were functionally validated in mCRC-PDX models and PDX derived organoids.

Results: In total 15.095 phosphorylated peptides derived from 4236 proteins were detected, including 255 phosphorylated kinases and 3073 (p)-tyrosine sites. Among other kinase ERBB2, EGFR, INSR and MET were identified as hyperactive driver kinases in cetuximab-resistant models. Sensitivity to inhibition of driver kinases alone or in combination with EGFR was confirmed in corresponding PDX-derived organoids. Group-based analysis identified potential biomarkers for response to cetuximab

Conclusion: Our findings improve the understanding of signalling pathways in mCRC and provide proof-of-principle of using phosphoproteomics for identification of driver kinases and patient stratification for individualised treatment.

Keywords: Phosphoproteomics, metastatic colorectal cancer, precision medicine, patient stratification, kinase profiling, (p)-tyrosine immunoprecipitation

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Identifying allergenic peptides presented class I major histocompatibility complex molecules on keratinocytes

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Background
In the class I antigen processing pathway major histocompatibility complex molecules (MHC-I) provide the immune system with a snapshot of the cellular proteome by selecting and presenting a selection of protein fragments, known as peptides, at the surface most cells. Collectively, the population of peptides is called the peptidome.
The role of MHC-I molecules in allergic contact sensitisation by chemical sensitizers such as 2,4-dinitro-1-chlorobenzene (DNCB) is unknown. However, DNCB has been shown to modify proteins directly in a process called haptenation, and may therefore modulate the nature of both the cellular proteome and the MHC-I peptidome. Here, we investigated the effect of DNCB on the MHC-I peptidome of keratinocytes using mass spectrometry proteomics.
Method
HaCaT keratinocyte cells were cultured in ether the presence or absence of DNCB and MHC-I molecules were isolated from lysates using immunoaffinity purification. The peptidome was isolated via HPLC and analysed using LC-MS/MS mass spectrometry (Thermo Orbitrap Fusion) and peptide identification was performed with a 1% false discovery rate with MaxQuant.
Results
We have characterised an initial HaCaT peptidome of over 1,000 peptides, identifying 20 peptides only seen in the presence of DNCB. We will probe the mechanism of DNCB specific differences in the peptidome using further mass spectrometry assays of the cellular proteome.
Conclusions
We next aim to identify peptides presented only in the presence of DNCB capable of stimulating an immune response.

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Comparison of MALDI imaging sample preparation/DESI imaging for multimodal MSI of Pre-clinical Breast Cancer

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The tissue sections were analyzed firstly by MALDI MSI using a SYNAPT G2-Si mass spectrometer with a MALDI source operating with a solid-state diode-pumped ND:YAG laser using a repetition rate of 1 KHz. The MALDI first sample preparation used was with CHCA in MeCH/Water. Consecutive tissues were then analyzed by DESI MSI, using a modified Prosolia source, directly mounted onto the SYNAPT G2-Si.
Normal breast tissue is mammary fat pad, with a high presence of triglycerides (TG). With DESI control tissue datasets, the highest signals were generated by the triglyceride molecules directly from the tissue sections i.e. m/z 879.74 (TG(54:6))H+ or (TG(52:3))Na+ and 853.73 (TG(52:5))H+ or (TG(50:2))Na+. However the molecular profiles changed considerably in the breast tumor samples, with an increase in intensity of signal for the detection of phosphatidylcholine (PC), i.e. m/z 770.51 (PC(32:1))K+ and 798.54 (PC(34:1))K+. This was not observed with the datasets generated by MALDI MSI. Indeed, with MALDI triglyceride molecules were clearly shown in both normal or cancerous tissue under the sample preparation conditions used. The observed difference in lipid profiles using MALDI between the tissue types were more subtle and were found to be related to differences in phospholipids. For example m/z 808.58 (PC(36:2))Na+ was less abundant in the tumor whereas m/z 772.52 (PC(32:0))K+ was more intense in the tumor sample.
Because of the lack of TGs in the MALDI MSI datasets, a second sample preparation was tested using CHCA in MeOH/Water. In this instance the presence of TGs was clearly observed in the control tissue section as well as the fatty part of the tumor bearing tissue section.
A third MALDI sample preparation using DHB in MeOH/Water will be tested to evaluate the nature of the lipids class ionized and to further examine the complementary nature of MALDI and DESI MSI datasets.

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Membrane proteome of failing heart. Combined forces of trypsin and cyanogen bromide.

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Background
Integral membrane proteins (IMPs) execute many essential functions and represent highly attractive drug targets. Our knowledge of their expression, structure and function is, however, quite limited. Although coded by roughly 25 percent of mammalian genes, IMPs are underrepresented in standard proteomic analyses. Their amphipathy, low expression, and absence of trypsin cleavage sites in their transmembrane segments are to be blamed. Current proteomic strategies either solubilize IMPs with the help of detergents or target only their accessible hydrophilic extra-membrane segments.

Methods
To profile the membrane proteome of heart muscle we employed an alternative strategy aimed specifically at hydrophobic transmembrane segments of IMPs. Our hpTC (high pH-Trypsin-CNBr) approach is based on a pioneering work of Blackler & Wu. This strategy solves the problem of abundant non-membrane contaminants in isolated membrane fractions by degradation and elimination of all non-membrane proteins and also extra-membrane domains of IMPs. Transmembrane segments of IMPs protected from the protease activity by phospholipid bilayer are then solubilized, re-digested with CNBr and delipidated prior LC-MS/MS analysis.

Results
We employed the hpTC method for the analysis of myocardial IMPs in a rat model of chronic heart failure. We identified 315 IMPs. Most of the IMPs were identified by peptides that overlaped with predicted transmembrane segments. In parallel, we performed a conventional proteomic analysis of whole tissue lysate of identical heart samples. We combined the data from both experiments and compiled an extensive dataset containing almost 600 cardiac IMPs.

Conclusions
Using two complementary approaches we identified almost 600 myocardial IMPs in a rat model of chronic heart failure. The dataset can be further expanded by implementation of another parallel strategy, namely glycopeptide enrichment.

Keywords
Integral membrane proteins, cyanogen bromide, myocardium, heart, heart failure

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IMPLEMENATION OF A NOVEL SCANNING QUADRUPOLE DIA ACQUISITION METHOD FOR DESI IMAGING

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Here we have assessed the applicability of this new method and optimisation of settings for a DESI imaging analysis. The Sonar method for DESI imaging consisted of two alternating functions. In both cases the quadrupole was scanned multiple times across the mass range with a pre set quadrupole window. In the first function (precursor function) the collision energy was fixed at 6ev, in the second function collision energy was applied to fragment the ions (MS/MS function). The functions alternated between pixels to generate images of precursors and of fragments in a single experiment. The precursor and MS/MS functions were subsequently time aligned to relate the fragments to precursors for identification of multiple species from a single imaging run.

Proof of concept experiments have been performed analysing a mouse brain tissue section in negative mode scanning the quadrupole from m/z 750-950 with a quad window of 8 Da. For the MS/MS function the collision energy was fixed at 30eV. Reviewing the data with Driftscope and HDImaging a number of time aligned precursors / fragments could be identified. One example was a PS(18:0_22:6) (m/z 834.52), from which fragments of the neutral losses of serine(m/z 747.49), the sn1 and sn2 RCOOH groups + serine (m/z 463.23 and m/z 419.26) were observed as well as the sn1 / sn2 RCOO- ions (m/z 327.23 and 283.26). In addition to the time aligned nature of the precursor/ fragment spectra, the spatial distribution of the precursors and fragments in the imaging data could also be used to further refine the precursor fragment assignments. Further work will be performed to optimise the quad settings to maximise the number of identifications and sensitivity / specificity.

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Optimization Of SONAR Collision Energy Ramps for Different Molecule Classes

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Initial experiments were carried out to determine optimum collision energy ramps using one quadrupole mass range, for example m/z 400 – 900 for analysis of complex peptide mixtures and m/z 500 – 1200 for small molecule applications. These were carried out in a systematic manner, i.e. by altering the starting and ending collision energy values in 5 V steps. Optimum ramps, based upon peptide identification rates and feature identifications, showed that ramps of 14 to 40V for proteomic and 20 to 50 V (+ve) and 25 to 55 V (-ve) for small molecules were appropriate values. However, the potential to further optimize collision energy ramps, with the aim of maximizing coverage, based upon precursor m/z and retention time is apparent when analyzing the identified precursors from the injection of 6 µg K562 cell line onto a 300 micron ID column. It is apparent there are distinct retention time regions where SONAR experiments would potentially benefit from using multiple quadrupole m/z ranges and collision energies, i.e. retention order, dependent. The analysis of complex proteomics samples show that the multi-step method has the potential to increase coverage and subsequent quantitation by > 20%. We also applied this same methodology to small molecule (lipidomics) experiments, increasing the feature identification rate significantly and improving the qualitative information by extraction of analyte class information based upon neutral loss or product ion extraction.

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Top-Down Proteomics identifies specific post-translational O-mycoloylations, which target Outer Membrane Proteins to the mycomembrane

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Background
Top-down mass spectrometry is emerging as a complementary tool to identify and characterize combinations of post-translational modifications (PTMs), truncations, splicing events and/or mutations. The corresponding protein isoforms, or proteoforms, can indeed display different activity, half-life, immunogenicity, hence the importance to fully characterize them.
The outer membranes (OMs) of members of the Corynebacteriales bacterial order, also called mycomembranes, harbor mycolic acids and unusual outer membrane proteins (OMPs), including those with α-helical structure. The signals that allow precursors of such proteins to be targeted to the mycomembrane have not been characterized so far.

Methods
We used a combination of top-down mass spectrometry, nuclear magnetic resonance spectroscopy and site-directed mutagenesis.

Results
We determined the proteoform repertoire of outer membrane proteins (OMP) from Corynebacterium glutamicum. We first investigated the partitioning of endogenous and recombinant PorA, PorH, PorB and PorC between bacterial compartments and demonstrated that they were both imported in the mycomembrane and secreted in the extracellular medium.
the analysis of endogenous and recombinant OMPs revealed specific and well-conserved PTMs, including O-mycoloylation, phosphorylation, pyroglutamylation and N-formylation, for mycomembrane-associated and secreted OMPs.

Conclusions
We found that the O-mycoloylation was essential for targeting to the mycomembrane. Furthermore, sequence analysis of O-acylation sites from C. glutamicum OMP and two eukaryotic proteins revealed unique patterns, suggesting that these PTMs have evolved throughout the kingdoms to guide membrane proteins towards a specific cell compartment.

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Keywords
Top-down Proteomics, Proteoforms, Acylation, Mycomembrane, Membrane Proteins

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Protein signature for endometrial cancer diagnosis in exosome-like vesicles of uterine aspirates

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Background

Endometrial cancer (EC) accounts for more than 10,000 deaths per year in the US alone. EC is divided into the more common estrogen dependent type 1 and the less common but more aggressive estrogen independent type2. There is an urgent need to develop non-invasive tests that can provide early detection of EC and that can discriminate EC subtypes. This study focuses on the identification of protein markers in exosome-like vesicles (ELVs) isolated from uterine aspirates. Uterine aspirates are collected by a minimally invasive procedure and it represents the ideal body fluid since it is the closest to the neoplasic endometrium cells.

Methods

Based on a previous discovery mass spectrometry study 54 protein candidates were selected for a targeted validation. A total of 86 unique peptides matching the proteins of interest were monitored by selected reaction monitoring (SRM) in ELVs isolated from human uterine aspirates. Isotopically-labelled peptides were spiked in each sample as peptide standards, and protein quantitation was performed using a QTRAP 5500 Sciex instrument.

The study cohort with a total of 107 patients included 3 age-matched groups: type 1 EC (n=45) EC, type 2 EC (n=21) and healthy individuals (n=41).

Results

Our targeted mass spectrometry approach confirmed that ELVs from uterine aspirates contain proteins that can discriminate between cancer patients and healthy individuals. More importantly, a 2-protein signature improves this capacity to discriminate healthy from EC patients (ROC AUC=0.935). This protein signature can detect endometrial cancer independently of the cancer type. In addition, we also report a new protein signature that can differentiate type 1 versus type 2 EC (ROC AUC=0.932). This study has important implications in early detection of EC and in patient stratification.

Conclusion

A targeted mass spectrometry approach defines protein signatures for endometrial cancer diagnosis in uterine aspirates.

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Combining heavy-methyl SILAC labelling with targeted MS to profile the dynamic histone methyl-proteome.

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Background. Histone post-translational modifications (PMTs) play a key role in the regulation of DNA-based processes by providing dockings sites for the recruitment of histone modifiers, chromatin remodelers and transcription factors that synergise to regulate gene expression. Mass Spectrometry (MS) has been successfully employed for the unbiased discovery of histone PTMs; however, the characterisation of less frequent histone methylations remains challenging. In this study, we used an integrated analytical strategy which couples heavy-methyl (hm)SILAC labelling, multiple-protease digestion and MS-targeted methods to discover and quantify histone methylations, with a special focus on Arginine-(R)-methyl-sites.
Methods. Histone peptides obtained from digestion with different proteases (ArgC, LysC and LysArgNase) were analysed on a last generation Q-Exactive mass spectrometer. Acquired spectra were processed using the MaxQuant (MQ) software, to assign methylation sites that were subsequently validated using a post-acquisition Perl-based pipeline that enables automatic search for hm-doublets at the MS1 level. Dynamic changes of histone methyl-peptides were profiled using the intensity of (hm)SILAC pairs in parallel reaction monitoring (PRM) experiments, using the Skyline software.
Results. This combined approach led to the identification of various novel methyl-sites on histones, comprising non-conventional aminoacids (D, E, H, Q) in addition to the more classical K/R residues. Noteworthy, we also found novel methylations on both H2A and H2B isoforms and linker Histone H1 variants. Through PRM experiments we also profiled methylation changes at various R-sites upon the genetic and pharmacological inhibition of protein arginine methyl-transferases (PRMTs), in order to systematically assess the molecular effect of this class of histone modifiers on chromatin.
Conclusions. Our combined MS-approach maximizes the specificity and sensitivity of histone methyl-sites assignment. Furthermore, the application of targeted MS to histone modification analysis is prone to clinical applications for the multiplex profiling of sets of relevant PTMs in patient samples.

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Identification of nephropathy predictive markers in urine from children affected by type-1 diabetes

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Background
Despite research progresses, the chance to accurately predict the risk for diabetic nephropathy (DN) is still poor. Although micro-albuminuria (which is detected only 10-15 years after the diabetes onset) remains the best available marker, it is not such a strong DN predictor. Only 30% of patients with micro-albuminuria, in fact, develop proteinuria, while in some patients it spontaneously regresses to normo-albuminuria. Our goal is to identify new predictive tools of nephropathy starting from urine, which is easily available and directly related to kidney.

Methods
Urine from children with recent type-1 diabetes diagnosis and age-matched healthy donors were collected and subjected to proteomic and lipidomic analysis. Validation by Western Blotting and ELISA was performed on different cohorts of type-1 diabetes patients.

Results
Our attention was focused on few potential biomarkers which can be predictive of renal dysfunction associated with type-1 diabetes.
Zinc-alpha 2 glycoprotein (a lipid-mobilizing factor) and prostaglandin-H2 D-isomerase were significantly over-expressed in diabetic children compared to healthy donors and in adults with DN compared to adults without DN. Also arylsulfatase A and β-galactosidase showed a similar behavior when comparing diabetic children versus healthy donors and adults with DN versus adults without DN, highlighting a dysregulation of ceramide production pathway. In addition, these data were supported by the urinary lipidomic analysis which showed a significant increase of ceramides and hexoseceramides, the downstream metabolites of the found up-regulated enzymes. Our results are in line with what was already described, since ceramide was demonstrated to be involved in renal fibrosis by enhancing extra-cellular matrix production, glomerular injury, proteinuria and apoptosis.

Conclusions
This study, combining proteomics and lipidomics, may contribute to identify markers of early worsening of renal function in type-1 diabetes
Keywords
urinary predictive markers; nephropathy; proteomics; lipidomics; mass spectrometry

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A TiO2 based simultaneous multiple PTMomics enrichment strategy

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Diverse protein post-translational modifications (PTMs) in proteins form complex combinatorial patterns to regulate the protein function and biological processes in a fine-tuning manner. Reversible phosphorylation, cysteines (Cys) modification, and N-linked glycosylation are well annotated PTMs essentially involved in cellular signaling pathways of pro-inflammatory cytokines, which can induce beta cell death and diabetes. In this study, we developed a comprehensive strategy for assessment of the proteome and multiple PTMomes: the TiO2-based simultaneous enrichment of reversibly modified Cys (rmCys) peptides, Phosphopeptides, and sialylated N-linked Glycopeptides (TiCPG) strategy, which enables one-step enrichment of peptides with three different PTMs from very low amount of sample, and largely minimizes sample loss. We applied this strategy to quantitatively study the change of these three PTMs in INS-1E cells subject to interferon-γ and tumor necrosis factor-α (IFN-γ/TNF-α) co-stimulation. It led to the identification of 27320 phosphopeptides (18762 phosphosites), 17634 rmCys peptides (12719 rmCys sites) and 4718 formerly Sialylated N-linked glycopeptides (2476 Sialylated N-linked glycosylation sites) from 7549 proteins with high specificity in the enriched fractions. In addition, 36060 unique peptides from 6396 proteins were identified from the non-modified fractions. Two or more different PTMs co-existed on 3457 proteins. Significant regulation was found on 100 proteins at the total protein level, while much higher degree of regulation was identified from 1490 proteins at PTMs level. The three PTMs were co-regulated in proteins, but demonstrated differential spatial and temporal patterns related to protein cellular localization and function in the time course of cytokines stimulation. Cytokines stimulation could induce the expression and change of three PTMs in proteins related to STATs,NF-κB, NOS2 signalings,receptors and immune related membrane-proteins. These processes together would initiate beta cell apoptosis. Overall, the TiCPG strategy is proved as a straight forward and powerful tool for the simultaneous characterization of multiple PTMs in biological or clinical applications.

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Identification and validation of protein biomarkers for homologous recombination deficiency in breast cancer

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Background
Triple negative breast cancers show very poor prognosis, with chemotherapy as the only treatment option. Because of a BRCA1 mutation and thus deficiency in homologous recombination (HR), a subgroup of these tumors is sensitive to DNA damaging drugs or blocking of non-homologous end joining via PARP inhibition. Therefore, the identification of HR deficient breast cancers via biomarkers is of importance for personalized therapy.

Methods
In a pilot experiment we used three biopsies each from HR-proficient or HR-deficient patient-derived xenograft (PDX) models, either untreated or 24h after cisplatin treatment. Global protein expression was profiled via label-free GeLC-MS/MS and changes in phosphorylation were examined using TiOx based phosphopeptide enrichment and single-shot LC-MS/MS (label-free).

Results
In total, we measured 8764 proteins and 6613 phosphopeptides with 6266 phosphosites. Samples cluster according to origin and treatment, and depending on the status of HR, we could identify differentially regulated (phospho)proteins. GO-term analysis of candidates upregulated in HR deficient samples revealed processes that are shared for both cisplatin treated and untreated tumors. These candidates are involved in processes such as DNA replication, DNA duplex unwinding and DNA geometric change. They seem to be important in the context of HR deficiency and are possible biomarkers.

Conclusions
Our pilot data point to the feasibility of detecting biomarkers for HR deficiency in breast cancer, also in the untreated situation. We will now expand our sample set of PDX models with known status of HR. Moreover, the predictive potential of candidate markers will be validated with in vivo intervention studies using PARP inhibitors.

Keywords
triple negative breast cancer, BRCA1, patient-derived xenograft (PDX) models, LC-MS/MS, (phospho)protein biomarkers

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ON-LINE IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY USING MAGNETIC BEADS FOR PROTEIN BIOMARKER ANALYSIS. TRANSTHYRETIN IN FAP-I.

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Background
Transthyretin (TTR) is a homotetrameric protein known to misfold and aggregate as stable insoluble fibrils causing different types of familial amyloidotic polyneuropathies. Among them, FAP-I, which presents a single amino acid substitution of valine for methionine at position 30 of the sequence (Met 30), is the most common. Traditional methods used to analyse the proteoforms of TTR in serum samples require an off-line sample pretreatment before separation and characterization by LC-MS.

Methods
In this study, TTR from human serum samples was immunoextracted, preconcentrated, detected and characterized by off-line and on-line IA-SPE-CE-MS using Affiamino magnetic beads derivatised with a polyclonal antibody (IA-MBs). Analysis of TTR from control and FAP-I serum samples was investigated.

Results
Identification and quantitation of the relative abundance of the 6 most abundant TTR proteoforms was achieved by the off-line IA-SPE and CE-MS. For on-line IA-SPE-CE-MS, under the optimised conditions with standards, IA sorbent lifetime (>20 analyses/day) and repeatability (2.9 and 4.3 % RSD for migration times and peak areas) were good. The method was linear between 5- 25 µg·mL-1 and limit of detection was around 1 µg·mL-1 (25 times lower than by CE-MS, 25 µg·mL-1). In both cases, in addition to the main normal TTR proteoforms detected in serum samples from healthy controls, the main mutant proteoforms (TTR-Cys (Met30) and Free-TTR (Met30)) were detected in FAP-I patients.

Conclusions
The developed on-line IA-SPE-CE-MS method for purification, separation and characterization of TTR from serum samples allowed a reliable, sensitive, reproducible and reusable screening tool for FAP-I. MBs offer a powerful alternative to expand the applicability of on-line IA-SPE-CE and an excellent opportunity to engage unskilled operators.

Keywords
capillary electrophoresis / magnetic beads / mass spectrometry / on-line immunopurification / transthyretin

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Composition and dynamics of the myddosome during the innate immune response

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Background: Critical for the innate immune response, the myddosome is a complex of proteins characterized by the presence of myeloid differentiation factor 88 (Myd88). The myddosome acts to transfer signals from the toll-like receptor (TLR) proteins to TNF receptor-associated factor 6 (TRAF6). In addition to Myd88, the myddosome contains multiple copies of the interleukin-1 receptor associated kinases (IRAK) 2 and 4 and is most often theorized to form following stimulation of the TLR proteins.

Methods: Using affinity purification – mass spectrometry analysis (AP-MS), we identified the Myd88-associated proteins in mouse immortalized bone marrow-derived macrophages before and after lipopolysaccharide (LPS) treatment. We examined the mechanisms that regulate the activity of the myddosome using AP-MS followed by phosphopeptide enrichment with MS/MS analysis to identify possible phosphorylation sites and to evaluate the role of phosphorylation on myddosome activity. We used SILAC with AP-MS to examine myddosome assembly and protein phosphorylation during a time course.

Results: Prior to LPS treatment, we found the stable association of Myd88 to IRAKs 2 and 4 along with the inhibitory factors IRAK3, TOLLIP, Socs5 and Sarm1. Following one-hour LPS treatment, the inhibitory factors were lost and Myd88 associated to the downstream effector proteins, including TRAF6.

Conclusions: In contrast to the prevalent model, our results suggest that the cellular myddosome is partially preassembled and only activates upon stimulation.

Keywords: innate immunity, Toll-like receptors, myddosome, AP-MS, quantitative proteomics

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Comparative proteomics of dying and surviving cancer cells pinpoints drug targets and reveals essential proteins

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Background:
Chemotherapeutics cause the detachment and death of adherent cancer cells. When studying the proteome changes to determine the protein target and mechanism of action of anticancer drugs, the still-attached cells are usually used, while detached cells are considered to lack structural integrity and are discarded. However, when cultured in fresh media, many such cells can recover and regrow, thus their proteome may contain valuable information for studying drug mechanism and investigating cell death and survival.
Methods:
To test this hypothesis, HCT-116, A375 and RKO cells were treated with 5-fluorouracil, methotrexate and paclitaxel, attached (50%) and detached (50%) cells were collected separately and their proteomes were analyzed by LC-MS with a 90 min gradient on a Q Exactive Plus mass spectrometer.
Results:
The attached and detached cell proteomes differed very significantly; yet drug targets were regulated in the detached cells as much as in attached cells. Combined detached and attached cell data provided a more accurate deconvolution of drug targets than any of them alone. Six proteins differentially expressed regardless of the treatment and cell type were targeted by siRNA, based on the hypothesis that such proteins might be key general players in cell death or survival processes. Knocking down USP11, CTTN, ACAA2 and EIF4H had anti-proliferative effects, while targeting UHRF1 additionally sensitized the cells to the anticancer drugs, and knocking down RNF-40 conferred cell survival against the chemotherapeutics. Downregulation of ribosomal proteins was found to be a survival feature in 5-fluorouracil treatment.
Conclusions:
The combination of proteomic data from attached and detached cells has an advantage in deconvolution of the targets of anticancer drugs. We identified several proteins potentially involved in general mechanisms of cell death and survival. Analysis of detached cells should become a standard practice in expression proteomics.
Keywords: cell death; chemotherapeutic; drug mechanism; mass spectrometry; survival

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Beta amyrin from the endophytic fungi Fusarium equiseti induce cell death in colon cancer cells.

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Background
Endophytic fungi are a precious resource of anticancer compounds. Derivatives of β amyrin, a penta triterpenoid are reported to have anticancer activities. Many plants and fungi are capable of producing amyrin. Several studies are underway. Colon cancer has high incidence rates and development of an effectual drug is a compelling emergency.
Methods
Fusarium equiseti was cultured in liquid medium, potato dextrose broth for 90 days. Secondary metabolites were isolated and purified by thin layer and column chromatography. Structural elucidation were carried out by infra red spectroscopy and liquid chromatography mass spectrum. Colon cancer cell line, SW620 was treated with different concentrations of the purified compound (β amyrin) and the cytotoxic effects were evaluated. Cell growth was measured by alamar assay, cell cycle arrest was assayed using flow cytometry and the mechanism of cell cycle arrest was investigated through Western blotting.
Results
After 48 h β amyrin treatment, the growth of SW620 colon cancer cell line was significantly inhibited in a dose dependent manner with IC50 value of 164.52 µg/ml. The population of SW 620 cells at sub G0 phase was increased by β amyrin at 100 µg/ml with a concomitant decrease in the G0-G1, S and G2M phase when compared to the control population in cell cycle analysis. p53 protein was expressed in Western blot analysis suggesting the possible mechanism of action of the compound as activation of the p53 pathway in cancer cells, leading to cell cycle arrest and apoptosis.
Conclusions
Beta amyrin, from the endophytic fungi Fusarium equiseti is capable of inducing cell death indicating the possible development of drug against colon cancer. Beta amyrin is a very expensive drug. Fungal endophyte can serve as an alternative for large scale production of the compound by cost-effective methods.
Keywords
Endophytic fungi, beta-amyrin, colon cancer, flow cytometry, Western blotting.

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Novel LC-MS assay unveils an acute decrease in serum sRAGE levels after cigarette smoking

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Background: The soluble Receptor of Advanced Glycation End-products (sRAGE) is a decoy receptor for various pro-inflammatory proteins and is primarily expressed in the lungs. sRAGE is considered to be one of the most promising COPD biomarker candidates based on data from various (large-scale) biomarker studies, which all used one particular ELISA for sRAGE quantification. Here we describe a novel approach for sRAGE quantification using LC-MS which was employed to study the effect of acute smoking on sRAGE levels.

Methods: sRAGE was quantified with a novel immunoaffinity UPLC-MRM/MS method in the range of 0.1-10 ng/mL using 50 µL of serum. The method was validated according to FDA guidelines, and was compared with the R&D Systems DuoSet ELISA. The method was applied to samples from a study on the acute effects of smoking in young individuals that are susceptible or non-susceptible towards developing COPD (based on family history) as well as COPD patients and age-matched (non-susceptible) controls. Samples were collected after two days of smoking cessation and also two hours after smoking three cigarettes within one hour.

Results: Comparison between LC-MS and ELISA data revealed a substantial correlation between both methods, yet with a large proportional bias. With any antibody-based method, levels are easily affected by interfering ligands, and such interferences may be investigated following our analytical approach. Furthermore, results of the acute smoking study showed that sRAGE levels decreased significantly in all groups after smoking. Therefore, these data may provide new insights in the role of sRAGE in COPD, but also indicate that smoking prior to blood sampling may lead to (artificially) decreased sRAGE levels.

Conclusions: Our work reports a complementary method for quantification of sRAGE which can be used to provide new insights in the role and value of sRAGE as a biomarker for COPD.

Keywords: COPD, Biomarker, LC-MS, Quantitation

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ProLiPALS: Proteomics of Lymphocytes from Parkinson's disease and Amyotrophic Lateral Sclerosis patients.

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Background
Parkinson's Disease (PD) and Amyotrophic Lateral Sclerosis (ALS) are neurodegenerative disorders whose pathogenesis is poorly understood. Nevertheless, PD and ALS share common pathogenetic mechanisms. Indeed, the frequency of extrapyramidal symptoms in ALS patients is significantly higher than in the general population. To distinguish between general and disease-specific patterns of neurodegeneration, we performed a meta-analysis of the literature of all the proteomic investigations of PD and ALS (Monti et al., 2016). This analysis revealed some pathways associated uniquely to PD or ALS, as well as pathways associated to both diseases. To verify alterations retrieved by the meta-analysis and to find new potential molecular actors, we perform a two-dimensional electrophoresis (2-DE) analysis of lymphocytes.
Methods
Lymphocytes samples from 20 Riluzole-treated ALS patients, 20 de-novo ALS patients, 20 de-novo PD patients and 20 ALS patients with PD symptoms were collected. Total proteins were separated by 2-DE and we selected spots that were significantly altered in different groups. Eventually, we used them to discriminate groups of patients by a hierarchical cluster analysis and a principal component analysis (PCA).
Results
Form analysis of 2-DE gels, we first excluded proteins altered in ALS patients by the Riluzole treatment. Then, we identified 40 proteins significantly altered in ALS, PD or comorbid patients. We used them to make a hierarchical cluster analysis and PCA. The clustered heatmap allowed us to identify spots that discriminate groups of subjects; moreover, cumulative proportion of the first and second principal component justified 72% of variance among groups.
Conclusions
PD and ALS pathogenesis share some mechanisms and a high incidence of comorbidity may be explained by common altered pathways. The combination of systems biology and the proteomic analysis of samples coming from comorbid patients will allow us to highlight pathways responsible for the degeneration of different neuron populations.
Keywords
Lymphocytes, Two-dimensional electrophoresis

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ADAR-mediated RNA editing in mouse brain proteome

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Background

Adenosine-to-Inosine (A-to-I) RNA editing is a posttranscriptional modification catalyzed by ADAR enzymes. In most cases, it occurs in neural tissue, where, as a result of the modification, adenosine is converted to inosine in particular sites of RNA, some of them code amino acid substitutions. We present a bioinformatics study of this phenomenon in brain proteome from C57BL/6 mice using proteogenomic approach.

Methods

Deep shotgun proteomes of the mouse brain regions and acutely isolated cell cultures were taken from public repository (Sharma et al., Nat. Neuroscience, 2015). Edited protein sequence database was generated using the available datasets of genome-wide ADAR editing analysis and was used to identify edited peptides. All datasets were searched using MaxQuant and X!Tandem engines with a group-specific false discovery rate (FDR) for edited peptides.

Results

We identified 10 edited peptides in 8 proteins, such as glutamate receptors Gria2, Gria3, Gria4, neural proteins Flna, Cyfip2, Cadps, Cog3 and Ube2o. Four identified peptides belonged to AMPA glutamate receptor complex which played a significant role in excitatory synaptic transmission. Identification of RNA editing in these proteins at the proteome level was in good agreement with background works (Tomaselli et al., Cell Tissue Res., 2014).

Conclusions

The signal from peptides subjected to A-to-I editing was strongest in cortical and cerebellar granule neurons, the fact, which is in good correspondence with the background art. Thus, it was shown that a proteogenomic approach is a potential tool for the analysis of RNA editing at the shotgun proteome level.

Keywords

ADAR, RNA editing, shotgun proteomics, proteogenomics, neuroproteomics

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Integrated miRNA, mRNA and protein profiling of exosomes and their parental cells

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Background
Myeloid-derived suppressor cells (MDSC) accumulate in the tumour microenvironment where they suppress adaptive and innate immunity. MDSC release exosomes (~30nm in diameter), which have been shown to mediate MDSC suppressive activity. Inflammatory conditions increase the abundance and function of MDSC, facilitating tumour progression. The aim of this study was to interrogate the protein, mRNA and miRNA contents of murine MDSC and MDSC-derived exosomes collected under conventional and heightened inflammation.

Methods
The protein and RNA cargoes of biological replicates from matched MDSC and their released exosomes were determined by shotgun proteomics and next generation sequencing, respectively.

Results
A total of 1434 and 1256 proteins were identified in MDSC and their released exosomes from both inflammatory conditions, respectively. More than half (58%) of the identified proteins were shared between MDSC and their exosomes, and 371 proteins were found enriched in exosomes. Interestingly, most of these enriched proteins are annotated by GO as associated with cell surface. Both miRNAs and mRNAs were shown to be present in exosomes shed under both inflammatory conditions. A total of 1453 miRNAs and 40433 mRNA transcript isoforms were confidently identified and used for further quantitative comparisons. Notably, our miRNA analysis provided the identification of 624 novel miRNAs. We observed that 85% of the proteins identified in exosomes were accompanied by their corresponding mRNA transcript isoforms. Moreover, the functions of several proteins, mRNAs and miRNAs identified are consistent with MDSC suppressive activity, suggesting a potential mechanistic redundancy.

Conclusions
This study represents, to the best of our knowledge, the first report providing evidence that MDSC-derived exosomes carry proteins, mRNAs and miRNAs with different qualitative and quantitative profiles than that of their parental cells. The differential content of proteins and RNAs suggests that MDSC and their exosomes may mediate some distinct immune suppressive functions.

Keywords
Exosomes, MDSC, mRNA, miRNA

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Quantitative Characterization of Hepatic Cytochrome P450 and UDP-Glucuronosyltransferase Enzymes: Evaluation against Catalytic Activity

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Background: Quantitative data of cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) enzymes are important in identifying metabolic pathways and predicting drug clearance and drug-drug interactions using physiologically-based pharmacokinetic models. Various proteomic methodologies are used to quantify these enzymes, with reports pointing out a large level of variation in expression, attributed to different sources. This study aimed to evaluate the quality of CYP and UGT quantitative proteomic data used in pharmacokinetic research against specific catalytic activity.

Methods: Four CYPs (CYP1A2, 2B6, 2D6, 3A4) and seven UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B15) were quantified using distinct proteomic methods and their catalytic activities were assessed in matching human liver samples (n=24). For UGT enzymes, the abundances were generated by two independent laboratories (stable isotope-labeled (SIL) standards or QconCAT). For CYP enzymes, two (QconCAT or label-free) methodologies were employed in the same laboratory. Catalytic activities to specific CYP and UGT substrates were used to validate abundance measurements. Several criteria were considered to improve abundance measurements: peptides/fragments selected for monitoring, protein content measurement.

Results: Unlike CYPs, there was little agreement between UGT abundance levels generated by different proteomic methods, except UGT1A1 (Rs=0.73, p<0.001; R2=0.30). Significant correlations between abundance and activity were demonstrated by SIL-based data, particularly for UGT1A1, 1A3, 1A4, and 2B7 (Rs=0.79-0.90, p<0.001; R2=0.69-0.79; n=59). For QconCAT, although correlations of abundance with activity for CYP enzymes were strong (Rs=0.65-0.82, p<0.001; R2=0.49-0.79; n=24), they were poor for UGTs, with moderate levels identified for UGT1A1, 1A3 and 2B7. Applying several optimization steps (selection of standard peptides, monitored fragments, optimization of protein content assay) significantly improved correlations in QconCAT data for six UGT enzymes (Rs=0.55-0.87, p<0.01; R2=0.48-0.73).

Conclusions: Enzyme abundance measurements should be validated against catalytic activity if available; several criteria can be considered to improve proteomic quantification.

Keywords: Human Liver, Cytochrome P450, UGT, Proteomics, Activity

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Differential Excretion of Uromodulin Peptides Indicates Early Changes in the Diabetic Kidney

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Background: Diabetes is the leading cause of kidney failure. Early functional and structural changes in the diabetic kidney may arise from dysregulated proteolytic activity. This activity is likely specific to certain proteases and their protein substrates. The resultant peptides generated within kidney may be excreted into the urine, providing a footprint of intrarenal proteolysis. However, the exact role of these players in early diabetic kidney disease is largely unknown.

Methods: We collected second-morning, midstream urines from 15 cases with type 1 diabetes and 15 age- and sex-matched controls. Urines normalized to creatinine underwent 10kDa-filter centrifugation to isolate naturally occurring peptides. Filtered peptides were then fractionated by strong cation exchange liquid chromatography and analyzed on a Q-Exactive mass spectrometer. MaxQuant was used for peptide/protein identification and label-free quantification. Peptide Extractor and Proteasix were used to examine protease activity based on amino acid sequences.

Results: A total of 6349 peptides from 750 proteins were quantified. We applied a stringent cut-off filter to identify and examine peptides found in all samples, resulting in 162 eligible peptides. Of the fifteen differentially excreted peptides (t test, $P < 0.05$), only five remained significant after Benjamini-Hochberg adjustment ($q < 0.05$), deriving from uromodulin and clusterin. Uromodulin peptides originated between a hepsin cleavage site and a GPI-anchor, containing a domain important in uromodulin polymerization. The sole clusterin peptide originated from the alpha chain near the nuclear localization signal. Peptide intensities were strongly correlated with glycated hemoglobin and blood glucose, and only moderately so with albumin/creatinine ratio.

Conclusions: Differences between adolescent cases with uncomplicated type 1 diabetes and healthy controls are reflected in the urinary peptidome before the development of microalbuminuria. Further investigation into dysregulated protease action on uromodulin and clusterin in hyperglycemic conditions may shed light onto early changes in the diabetic kidney.

Keywords: peptidomics, diabetes, proteases

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Quantitative profiling of glycerophospholipids during mouse and human macrophage differentiation using targeted mass spectrometry

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Background:Macrophage lipid metabolism plays a pivotal role in innate and adaptive immune responses. Previous studies have shown that this process plays a role in infections and contributes to the pathogenesis of diabetes, atherosclerosis, and other immunometabolic diseases. M1 macrophages, or classically activated macrophages, are key players in the defense against bacterial infections. M2 macrophages, or alternatively activated macrophages, are involved in anti-inflammatory responses.
Methods: We used the pattern of M1 and M2 macrophages to delineate the lipid composition by multiple reaction monitoring(MRM) method under macrophage differentiation process. The M1 or M2 macrophage marker gene expression was determined by the real-time qPCR measurement.
Results:We detected over 300 lipid molecules in mammalian macrophages, and we observed a striking shift in the composition of glycerophospholipids(GLs) from saturated and monounsaturated to polyunsaturated during human macrophage polarization. Moreover, M2 macrophages showed a higher level of lysophospholipids (lysoGLs) than did M1 macrophages. The lysoPI species increased in human and mouse M2 macrophages, suggesting that they may be involved in M2 macrophage polarization and anti-inflammatory processes.
Conclusions:These results indicate that lipids may play a role in the pro- and anti-inflammatory activities of macrophages and may be markers of the macrophage activation state.
Keywords:Macrophage differentiation / MRM / lipidomic

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Next-generation Proteomic Pipeline (NextPP) for Chromosome-based Proteomic Research Using GENCODE and neXtProt Databases

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Backgound: The human genome annotation in the encyclopedia of DNA elements project (GENCODE) database contains more alternative splicing variants (ASV) and non-coding gene sequences than the neXtProt database, has used for novel findings of protein variants. Before claiming novel findings, including missing proteins and novel ASVs, the Human Proteome Project (HPP) guidelines suggest considering extra explanations for isobaric modifications or single amino acid variants (SAAVs).
Method: To find novel ASVs and missing proteins while considering extra explanations for SAAVs, we suggest a streamlined pipeline with customized and concatenated databases from GENCODE and neXtProt with both false discovery rate (FDR) -filtering conditions at both the peptide and protein levels. First, we show that more stringent FDR filtering (<0.1% at the peptide level) is needed to claim novel findings.
Results: Using this filtering condition, four (DPYSL2-005, NCAM-013, MPRIP-003, and SYN2-001) of 27 novel ASVs with two or more unique peptides were commonly identified from five types of datasets related to human brain tissues: hippocampus (PXD0033695), cortex (PXD000067), another cortex, spinal cord, and fetal brain (PXD000561). The most of identified peptides of novel ASVs were mapped to novel exon insertions or alternative translations at 5'-untranslated region. In all, 17 of 46 novel ASVs were commonly identified from two datasets of testis tissues (PXD000561 and PXD002179). In addition, four missing proteins with two or more unique peptides, LYZL1 (from PXD002367), TOMM6L (from PXD002179), ARC (from PXD000067), and GRIK5 (from PXD000395), remained after the PE levels were verified from the latest version (release 2017 Jan) of the neXtProt database.
Conclusions: During the analysis of each dataset using our pipeline, as the next-generation proteomic search pipeline for C-HPP study, we also identified and considered SAAVs to claim findings of novel ASVs and missing proteins.
Keywords: C-HPP, Proteogenomics, Alternative splicing variant, Single amino acid variant, missing proteins

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Discovery of inflammatory signaling adaptors regulated by autophagy

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Genome-wide association studies have revealed that defects in autophagy are strongly associated with increased risk of chronic inflammatory diseases such as Crohn's disease and Lupus. Despite significant advances in our understanding of phenotypes associated with loss-of-function in autophagy, the mechanisms by which autophagy regulates inflammatory signaling remain unclear. To identify signaling adaptors that specifically mediate Atg16L1-dependent autophagy in an inflammatory context, we compared Bone Marrow Derived Macrophages (BMDMs) from wild type and Atg16L1 deficient mice, following an LPS treatment to stimulate Toll Like Receptor (TLR) signaling. Through a quantitative multiplexed proteomics assay and Tandem Mass Tagging (TMT) we confidently identified and quantified no less than 6,152 proteins but surprisingly observed that only a select set of proteins, including key autophagy players, were up-regulated rather than observing global protein accumulation. Further examination of clustered temporal profiles allowed us to distinguish between basal regulators that trended towards equalization upon LPS exposure and those that show exaggerated elevation at advanced inflammation stages. In addition, investigation of pathways common to all significant responders revealed that defective autophagy impacts specific processes related to turnover of ubiquitinated cargo targeted to the autophagosome. Taken together, we demonstrate that in this global comprehensive study we identified key autophagy components with high sensitivity while identifying relative changes in this complex pathway with high specificity in a global proteome.

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Quantitative dot blot analysis (QDB), a versatile high throughput immunoblot method for validating proteomics results

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Background
Lacking access to an affordable method of high throughput immunoblot analysis for daily use still remains a big challenge for scientists worldwide. Thus we proposed here Quantitative Dot Blot analysis (QDB) to meet this demand.
Methods
The QDB approach is based on the pre-made QDB Plates. The samples are applied directly to the membrane bottom of the individual units. The loaded membrane is then processed through steps of traditional immunoblot analysis to form immunocomplexes on the membrane before developed through a chemiluminescence reaction in a microplate reader for quantification. QDB analysis was tested by analyzing the tubulin content in mouse liver lysates compare to traditional Western Blot side by side. The application of QDB method was also evaluated at cellular level by measuring the expression level of P65 in p65-knockdown HEK293 cells. At tissue level, we measured protein expression level of CAPG in mouse prostate tissue lysates from 87 individual animals (n=87, 40 WT and 47 prostate cancer transgenic mice, age range 10 weeks to 24 weeks).
Results
For tubulin analysis, QDB yields a linear curve between 0 to 1µg of mouse liver lysate, R^2 =0.999. In comparison, the Western blot quantification yield a linear curve between 1 -12 µg mouse liver lysate (R^2 =0.85). For cellular level application, p65 expression level was measured in 71 individual clones with a normal distribution in a population level by statistical analysis. Furthermore, we were able to measure the absolute CAPG content in 87 prostate lysates with the recombinant CAPG standard.
Conclusion
QDB is a simple, high throughput, robust and reliable analysis approach. It is able to transform the current semi-quantitative immunoblot method into a true quantitative assay to provide more information with accuracy and reliability for proteomic research.
Key words
Quantitative dot plots, Western blot, high throughput, true quantitative

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Characterization and Quantification of CHO Host Cell Proteins during Monoclonal Antibody Purification by SWATH-MS

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Background
Residual host cell proteins (HCPs) impurity is one of the critical quality attributes (CQAs) in CHO-produced therapeutic monoclonal antibodies (mAbs). Antiserum-based immunoassays such as ELISA are widely used for HCP detection in industry. However, the limitation of the antiserum, raised against either total or a subset of HCPs, makes the assays less feasible to monitor individual HCPs. Recently, SWATH-MS has established itself as a powerful tool in various large-scale proteomics studies. With the features of wide dynamic range and fast acquisition speed, a sensitive, robust and effective SWATH-MS workflow was developed to monitor each individual HCP clearance in the process intermediates or the final product of mAb production.
Methods
Firstly, CHO-K1 whole cell lysate was analysed by data-dependent acquisition to generate a spectral ion library for SWATH-MS analysis. Secondly, two types of samples were analysed using SWATH-MS data acquisition, including (1) pre-mixed control samples with known ratios of pure mAb to residual HCPs and (2) downstream purification intermediates of a mAb product.
Results
SWATH-MS analysis of pre-mixed samples showed that up to 60% of the total identified 2413 proteins were accurately quantified with the quantitative fold change falling within the range of theoretical values ±20%. SWATH-MS analysis on mAb process intermediates identified and quantified up to 1465 HCPs across the samples. Subsequent spiking of the samples with known quantities of standard proteins enabled the absolute quantification of individual HCPs and resulted in a five-fold higher sensitivity than the previously reported detection limit.
Conclusions
We have demonstrated that SWATH-MS coupled with a good CHO-HCP ion library is able to identify and quantify the HCP impurities that are relatively unexplored to date. The workflow provides rapid and robust analytical support toward process development of biotherapeutics production to achieve optimal HCP clearance.

Keywords: mAb, host cell proteins, SWATH-MS analysis

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Proteomic insights into cancer-related extracellular proteolysis with cathepsin K

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Background

Cysteine cathepsins are lysosomal proteases from clan CA that gained much attention for their role in cancer development and progression. Among these cathepsin K has strong collagenolytic activity and participates in several physiological and pathological processes, especially when secreted in the extracellular space. Besides osteoclasts different cancer cells express significant amounts of cathepsin K and the use of selective inhibitors can significantly reduce the cancer-related osteolysis and bone metastasis burden. Cathepsin K could thus have an important role in the development of bone metastasis, but its molecular functions and proteolytic substrates remain poorly understood.

Methods

We established a mass spectrometry-based proteomic methodology where intact cells were treated with recombinant cathepsin K and the released extracellular proteins were identified by mass spectrometry. Our study included cancer cell lines MDA-MB-231 (breast cancer), HT-144 (melanoma), PC-3 (prostate cancer) and Saos-2 (osteosarcoma), known to metastasize or develop in bones.

Results

We identified several extracellular substrates of cathepsin K and the detailed bioinformatics analysis showed that the majority of them is well-conserved over several cancer cell lines. Among them were cell adhesion molecules (e.g. CD44, CD166) and specific cell surface receptors (e.g. TFR-1, PLXNB2), several of them already shown to have important roles in cancer development and progression.

Conclusions

Our results suggest that cathepsin K has important roles in cancer-related extracellular proteolysis and could have a major role in cancer progression and bone metastasis. Moreover, in the light of the recent failure of cathepsin K inhibitors in clinical trials the identification of its substrates in context of cancer could open new avenues for development of cathepsin K-based therapeutics.

Keywords

Extracellular proteolysis, cathepsin K, cancer

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Serum degradation analysis by MALDI/ToF: a new method and tool

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Background
Biological samples undergoing long term cryopreservation are used in retrospective studies for biomarker identification. Moreover, the handling of samples before freezing may affect their quality. The quality of source material must be assessed before any study. The peptidomic profile of a serum sample is characterized by some fibrinopeptide related signals, mainly fibrinopeptide A (fpA) and its degradation products, generated during coagulation.
Methods
The high susceptibility of fpA to degradation suggests its use as a quality indicator for cryopreserved serum samples. When properly preserved, they should mainly contain whole and little degraded forms of fpA. Samples exposed to a greater extent to chemico-physical injuries should show a greater presence of shorter peptides. We defined a mass spectrometry based method and developed a software tool to assess the integrity level of serum samples by evaluating their fibrinopeptide contents. The method also takes into account a serum sample of good quality, analyzed with the same procedure.
Results
The Serum Degradation Analysis software processes spectra to extract peak lists, which are then elaborated for noise reduction. Finally, fpA related peaks are compared in order to compute their overall abundance and the percent contribution of each peptide. If a reference spectrum is provided, overall abundances are expressed as percent of the reference ones. A score is assigned to spectra by taking into account both their overall fpA abundance and the ratio between abundances of more and less degraded forms. Elaboration is optimized by processing spectra in parallel on a multicore virtual server.
Conclusions
The proteomic analysis of serum samples is a typical approach to the study of several diseases, including cancer, which needs good quality specimens. The Serum Degradation Analysis software is an effective tool for the evaluation of the preservation level of serum samples (http://bioinformatics.hsanmartino.it/seradeg/).
Keywords
MALDI/ToF, serum quality, fibrinopeptide degradation, web server

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Colims: a distributed proteomics LIMS system

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One of the first points of failure in the structured capture and dissemination of proteomics data and results is encountered as early as how local data management is handled by research groups. Very often, no specialized software outside of a search engine and/or a quantification engine is used, and even if some form of aggregator or post-processing software is used, these existing (commercial or freely available) solutions lack structured, long-term data storage. This impacts reproducibility, and hinders quality control and data analysis.

Here, we present Colims, the next generation of ms_lims [1], a LIMS system for local data management, processing and analysis. Colims stores data on the spectral, PSM, protein and quantification level. Next to an exhaustive list of features extracted from the experimental results, Colims also stores the settings used to generate the data. This empowers users to have a memory of how the data was generated. The added focus on meta-data allows Colims to substantially help in enabling the comparison of different experimental results, keep a high level of quality assurance and dramatically cut down time required to export data into submission ready formats such as mzTab and pride XML. All of the data is furthermore quickly and efficiently retrievable from the relational database at the heart of the system.

Having learned from cloud implementations, Colims offloads computational heavy tasks to dedicated machines. This allows the storage and data repository to be locally or on separate servers, in house or off-site. Using a robust messaging server, Colims provides fast and high performance communication between the main controller and the users.

Next to managing data, Colims gives the lab tools to manage access and rights for users, collaborators and guests on the database, project and experiment level.

Project homepage: http://compomics.github.io/projects/colims.html

Keywords: Proteomics, LIMS, data storage, MaxQuant, PeptideShaker, Pride

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Development of a nonglycopeptide-based MRM strategy for screening of differential glycoproteins in hepatocellular carcinoma

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BACKGROUND
Qualitative and quantitative study of aberrant changes of glycoproteins in serum is particularly important for biomarker discovery. Direct identification and quantitation of glycoproteins in serum based on mass spectrometry (MS) still have been technically challenging due to the extraordinary complexity of the serum proteome. We propose an efficient glycoproteome multiple reaction monitoring mass spectrometry (MRM-MS) strategy monitoring of nonglycopeptides from N-glycoproteins.

METHODS
The nonglycopeptides released from hydrazide chemistry (HC)–captured glycoproteins were identified, selected and optimized as internal references for MRM quantification of glycoproteins in human carcinoma cell (HCC) serum. These nonglycopeptides were used for glycoprotein relative quantification in HCC serum with mTRAQ labeling and MRM-MS analysis. The results were validated by MRM-MS absolutely quantification and enzyme linked immunosorbent assay (ELISA) analysis.

RESULTS
The new MRM-MS quantification strategy based on nonglycopeptides was developed for glycoprotein quantification. The strategy has been proved to be feasible for glycoprotein quantification. A data set of 283 nonglycopeptides containing a series of optimized parameters that can be used for MRM-MS glycoprotein quantification in HCC serum was established. Combined the new strategy and mTRAQ labeling, a total of 100 glycoproteins in HCC and normal serum were relative quantified. And the quantitative accuracy of the nonglycopeptides for glycoproteins were well validated.

CONCLUSIONS
This efficient strategy provides a useful tool for glycoprotein quantification and broadens the application of MRM in quantitative glycoproteome. The nonglycopeptide data set with optimized transitions and parameters established in this research is valuable for the glycoprotein-biomarker quantification and exploration of new glycoprotein biomarkers in HCC serum.

KEYWORDS
Glycoprotein ， MRM ， Quantification ， Hepatocellular carcinoma

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NSCLC cells use increased cell-cell contacts to resist early TKI treatment

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Background
Targeted therapies against oncogenic receptor tyrosine kinases are showing promising results in the clinic. However, despite the initial response, most patients become resistant. Most research regarding resistance has been focused on acquired resistance occurring after an extensive time of treatment. This has resulted in the development of combination treatments. Yet, the question remains how cells are able to survive initial treatment. We hypothesize that early resistance to apoptosis will enable cells to develop any growth stimulating mechanism. Therapeutically, early inhibition of adaption could potentially prevent further resistance development.

Methods
A multi-omics approach was used, including mass spectrometry based proteomics, phospho-proteomics, kinomics and metabolomics, to gain more insight in regulated processes during early treatment. A panel of lung and breast cancer cell lines was characterized for their response to various drug treatments. Potential new drug targets were validated using knock-downs and co-inhibition treatments.

Results
Regardless of an initial drug response, drug sensitive cells rapidly adapt to targeted therapy. Within days they continue to proliferate, despite persisting target inhibition. Co-inhibition of well-known resistance pathways, for example mTOR activation, delays proliferation reoccurrence, but doesn’t prevent it, nor increases sensitivity to apoptosis.
Proteomics data shows an increase in abundance and activity of cytoskeleton and calcium signaling related proteins. In addition, cells show a distinct morphological change of increased cell-cell contact. We found that if cells were restricted from making these cell-cell contacts, they are no longer able to survive TKI treatment. Consequently, development of any resistance mechanism will be prevented.

Conclusions
Using a multi-omics approach combined with cellular methods we were able to shed light on the early response of sensitive cells upon targeted drug treatment. Their first action is the differentiation into a more endothelial morphology in order to resist apoptosis. This will enable further development of resistance.

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MALDI-TOF mass spectrometry on the track of sand fly life cycle

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Background
Blood-sucking females of phlebotomine sand flies represent well-known vectors of viruses, bacteria, and most importantly parasites of the genus Leishmania causing leishmaniasis, an emerging protozoan disease affecting about 350 million people in many countries worldwide. To ensure proper vector control and monitoring in epidemiological studies conducted in endemic areas a method for reliable species identification is highly desirable. The aim of the study was to evaluate the usefulness of MALDI-TOF MS for the identification of different life stages of sand flies and map the changes in their protein profiles during the whole life cycle with the focus on immature stages.
Methods
Larval, pupal and adult bodies of six different sand fly species were homogenized using a pestle in 25% formic acid, mixed with a MALDI matrix (sinapinic acid, 30 mg/ml in 60%ACN/0.3%TFA) and subjected to MALDI-TOF MS analysis.
Results
For larvae, the spectra were reproducible and easily distinguishable at the species level and the profiles were stable from the L2 to L4 developmental stages. Therefore only profiles of L4 stages were selected for the creation of juvenile reference database. The performance of the database was tested in a blind study where 123 specimens (from L2 to L4) of six different species were included. The test resulted in 92.7% correct identifications regardless the larval stage. Pupal spectra allow identification at species level as well and surprisingly, they did not change during metamorphosis as observed for other holometabolous insects. For adult females, the influence of age, blood and eggs on spectrum profile was also assessed.
Conclusions
The study indicates that MALDI-TOF MS is a suitable method for identifying not only adults, but also immature stages of phlebotomine sand flies.

Keywords
MALDI-TOF MS, species identification, sand fly, life cycle

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MRM Mass Spectrometry for quantification of Histone H3 variants PTM marks in Arabidopsis Thaliana

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Histone variants and their post-translational modifications (PTMs) play an important role in the developmental processes of animals and plants. For example, H3K27 and H3K36 methylation are associated with transcriptional activation and silencing respectively. With traditional method such as West blotting, it is difficult to identify and quantify the change of modifications in histone variants, especially in plants, since they have longer life span and similar properties. In this study, we set up a new MRM method using 20 standard histone H3.3 peptides. Combined with our previous studies, we were able to quantify the k27/36 combination modifications on histone H3.1 and H3.3 specifically in a single experiment. This method has been verified to be highly accurate and reproducible. Then we applied this method to investigate the double mutants' atxr5/6 (the histone H3 lysine-27 monomethyltransferase) in Arabidopsis thaliana. Our results showed that ATXR5/6 prefers to methylate histone H3.1 rather than histone H3.3, which was consistent with the previous report. To our surprise, we found that H3K27me1 was antagonized with H3K27ac.In the future research, this new method will provide some new insight on the relationship in different PTMs on histone H3K27/36.

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Characterising the role of protein kinase CK2 in regulating aluminium toxicity in yeast

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Background
Chronic environmental exposure to aluminium has been linked to cancers and neurodegeneration. Through a previous genome wide deletion study, using the model organism Saccharomyces cerevisiae, we have demonstrated that deletion of the catalytic subunit (CKA2) of the renowned tetrameric protein kinase CK2 results in a tolerant phenotype to Al3+. These findings provided the basis for our proteomic investigations into the mechanisms of Al3+ toxicity and detoxification.

Methods
Five strains of S. cerevisiae, including Al3+ sensitive BY4743, cka1Δ, ckb1Δ and ckb2 as well as Al3+ tolerant cka2Δ were treated in duplicate with 1.6 mM Al3+ in a time-course (0 to 16 hr). Reverse phase chromatography was used for creation of the spectral library which was generated with ProteinPilotTM 5.0 and searched against the yeast Uniprot 2016 database for protein identification. The spectral library was imported into PeakViewTM 2.1 and matched against SWATH-MS data for protein quantification. Statistical analysis of the differentially expressed proteins with a fold change cut-off of 1.5 was performed using Perseus 1.5.5.3.

Results
A total of 3196 proteins (1% FDR) were identified in this study and 2283 proteins were successfully quantified via SWATH-MS. In the Al3+ sensitive strains, Al3+ was found to damage the cell wall and plasma membrane, disrupts processes such as nucleotide synthesis and repair, amino acid metabolism, ribosome biogenesis and the anti-oxidant response. The key findings of the protective role of cka2Δ against Al3+ include overexpression of proteins in sulfur metabolism, lysine biosynthesis, anti-oxidants and the heat shock response.

Conclusions
These novel findings provide in-depth understanding of Al3+ toxicity and its detoxification, and have implications in finding solutions to Al3+-related problems such as neurodegenerative diseases and cancers.

Keywords: Aluminium toxicity, Protein kinase CK2, Proteomics, S. cerevisiae

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Screening of Mycobacterium tuberculosis 19kDa antigen proteoforms by Top-down and Bottom-up approaches

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Background

Post-translational modifications (PTMs) are essential processes conditioning the biophysical properties and biological activities of the vast majority of mature proteins. However, occurrence of several distinct PTMs on a same protein dramatically increases its molecular diversity. The comprehensive understanding of the functionalities resulting from any particular PTM association requires a highly challenging full structural description of the PTM combinations, which could play a key role in the study of virulence factors of infectious disease, such as tuberculosis.

Methods

Here, we report the in-depth exploration of the natural structural diversity of the Mycobacterium tuberculosis (Mtb) virulence associated 19kDa lipoglycoprotein antigen (LpqH) using a combination of top-down and bottom-up proteomics analyses by high-resolution mass spectrometry (HR-MS) coupled to liquid chromatography.

Results

Analyses of the purified Mtb LpqH protein allow us to uncover a complex repertoire of molecular species resulting from the intrinsically heterogeneous combination of lipidation and glycosylation, together with some truncations. Direct view on the co-occurring PTMs stoichiometry reveals the presence of functionally distinct LpqH lipidation states with different glycosylation degrees, and indicates that glycosylation is independent from lipidation. We were also able to identify an unsuspected phosphorylated form of the unprocessed preprolipoglycoprotein, without any glycosylation or acylation.

Conclusions

This work allowed the identification of about 130 distinct proteoforms of the 19kDa antigen from Mycobacterium tuberculosis, with multiple PTMs combinations. The identification of a novel unsuspected phosphorylated form of the unprocessed preprolipoglycoprotein, totally absent from the current lipoglycoprotein biogenesis pathway, provides new insights into the biogenesis and functional determinants of the mycobacterial lipoglycoprotein interacting with the host immune Pattern Recognition Receptors (PRRs).

References

Parra, J. Marcoux, J. et al. Scrutiny of Mycobacterium tuberculosis 19 kDa antigen proteoforms provides new insights in the lipoglycoprotein biogenesis paradigm. Sci. Rep. 7, 43682 (2017). doi: 10.1038/srep43682

Keywords

Tuberculosis; Top-down MS; Post-translational Modifications; Proteoforms.

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Uncovering the molecular underpinnings of NGLY1 disease through personalized proteomics and glycoproteomics profiling

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Background
N-glycanase deficiency is a rare genetic disorder with complex neurological manifestations including delayed development, seizures, and auditory neuropathy. N-glycanase, the protein encoded by the NGLY1 gene, regulates the endoplasmic-reticulum-associated degradation (ERAD) pathway through dynamic protein de-glycosylation. Systematic characterization of differential protein levels and glycosylation in the context of NGLY1 disease is lacking. Hence, the underlying mechanisms misregulated under N-glycanase deficiency remain poorly understood.

Methods
We cultured induced pluripotent stem cells (iPSCs) derived from a patient with distinct recessive loss-of-function NGLY1 mutations in each allele. Subsequent CRISPR/Cas9 correction was performed to produce wildtype NGLY1 alleles in these cells, allowing us to study the effect of clinical NGLY1 mutations whilst controlling for genetic background. We used shotgun liquid chromatography coupled to tandem mass spectrometry-based proteomics and data-independent targeted proteomics (SWATH-MS) to interrogate differences in the proteomes. Furthermore, we used a novel intact glycopeptide enrichment method followed by mass spectrometry to detect changes in respective glycoproteomes concurrent with N-glycanase deficiency.

Results
Our pipeline yields high-throughput quantitative characterization of patient-derived iPSCs – not only on the protein level, but also at the resolution of site-specific intact glycopeptides. Pilot differential expression analysis revealed that N-glycanase deficiency conferred higher levels of PGM1/PMM2 ($p < 0.05$, $\log FC > 1.5$), glycometabolism enzymes associated with other congenital disorders of glycosylation. Key proteins of the ERAD ubiquitin-proteasome machinery (UBXN4, PSMD10, RBX1, UBE2V2 etc.) also accumulated ($p < 0.1$, $\log FC > 1$) in double-mutant cells, suggesting misregulation of cytosolic protein degradation under N-glycanase deficiency. Moreover, glycoproteomics profiles showed differential sialylation and overexpression of site-specific N-GlcNAc as post-translational aberrations associated with NGLY1 disease.

Conclusions
We present a novel approach to study aberrant glycosylation and protein aggregation in patient-derived iPSCs. Our analysis provides the first proteomic and glycoproteomic characterization to uncover insights on NGLY1 disease etiology.

Keywords
NGLY1, rare disease, proteomics, glycoproteomics

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Analysis of human brain N-glycans by (PGC-)LC-ESI-MS using isotopically labeled standards

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Background:
N-glycosylation is the most complex and heterogeneous post translational modification. It is important for protein folding, stability and biological interactions of many proteins. The large number of possible isomers yielding from one glycan composition necessitates powerful separation techniques (porous graphitic carbon LC) and the use of defined standards combined with MS/MS.
Methods:
Proteins of different human brain tissue sections (as well as mouse brain) were purified, treated with PNGase F to release the N-linked glycans and reduced with sodium borohydride. The glycans were purified and directly subjected to a PGC-LC-ESI-MS system (QTOF instrument). Prior to the measurement defined isotopically labeled N-glycan standards were added to identify and quantify the isomers occurring. The incorporation of different numbers of heavy isotopes at varying positions ($^{13}C_6$ and $^{13}C_1$ galactose, reduction with sodium borodeuteride or deutero acetyl groups of GlcNAc) allowed the assembly of internal multiplex standards mixtures.
Results:
The major glycan was found to be identical in every brain section with the mass of a bisecting, fucosylated GnGn (proglycan nomenclature, www.proglycan.com). Interestingly the degree of sialylation varied in the different brain areas. All main isoforms could be identified and relatively quantified. Absolute quantification was furthermore achieved by the quantitation of one ^{13}C labelled standard by amino sugar analysis. This one internal standard allowed for the calculation of the other glycoforms by the use of instrument dependent correction factors (calculated ratios known from an equimolar standard mix [1])
Conclusion:
A powerful method for the analysis of N-glycans in complex tissue samples is presented. The use of different labeled standards allowed identification and quantification of glycan isoforms in a single MS-run.

[1] Grünwald-Gruber C, Thader A, Maresch D, Dalik T, Altmann F. Determination of true ratios of different N-glycan structures in electrospray ionization mass spectrometry. Anal Bioanal Chem. 2017 Apr 409(10):2519-2530

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Complementary tandem mass spectrometry approaches to distinguishing peptides containing amino acid isoforms

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Background
Recent improvements in high performance mass spectrometry and the progress in DNA sequencing led to a rapid development of a new research area called proteogenomics. One of its main objectives is searching for point mutations at the proteome level using customized protein databases generated from DNA or mRNA sequencing. However this search may confront a problem of identifying chemical modifications mimicking single amino acid substitutions. One of the most challenging is chemical modification of methione to isothreonine (homoserine) under commonly used alkylation reagents.
Methods
We evaluated the methods for distinguishing between synthetic peptides with threonine (Thr) and isothreonine (isoThr) residues. The evaluation included three MS/MS methods: higher energy collision (HCD), electron transfer dissociation (ETD), and recently introduced 2D UV-MS cold ion spectroscopy. One of the unique features of the latter method is high specificity of UV spectra to peptide spatial structure.
Results
Significant differences in fragmentation spectra of peptides containing Thr and isoThr have been revealed for all three MS/MS methods with 2D UV-MS being the most efficient one. Note also, that the highly confident Thr/isoThr peptide distinguishing was observed in both UV and UV photo-dissociation (UVPD) spectra obtained at specific wavelength. Importantly, this method can be successfully applied to peptides without strong light-absorbing chromophores in the sequence.
Conclusion
Peptide isoforms containing isoThr residue can be generated during the proteomic sample preparation and produce false positive identifications of genetically encoded peptide variants. Fragmentation pattern of these peptides can potentially reveal the presence of isomeric form of the residues, thus, allowing distinguishing between these two events. Among the three MS/MS methods evaluated in this study for Thr vs isoThr we found UVPD fragmentation being the most specific to the presence and location of isoThr in the sequences.
Keywords
2D UV-MS, UVPD, ETD, Isotheronine, Proteogenomics

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CSF analysis for Protein Biomarker Identification and Validation in Patients with CNS Lymphoma

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Early diagnosis and treatment of CNS lymphoma attenuates disease progression and neurologic deterioration. Then, new approaches for early detection based in identification of biomarkers are required, particularly for CNS lymphoma which cytologic examination is less than 50% sensitive than other cancers. In this study, we report a strategy for quantitative differential proteomic analysis that also includes large-scale quantitative identification of more than 500 proteins to identify the major CSF proteins which distinguish B-cell CNS lymphoma from benign conditions. Then, after the deep LC-MS/MS analysis, in order to generate and easy translation into the clinic, for the multiplex detection of these proteins, a kit based on color-coded bead suspension array has been designed and developed for the determination of tumoral infiltration in central nervous systems (Leptomeningeal Metastasis (LM)). Here, it will be presented the results obtained from the validation data set in a cohort of > 100 samples.

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Proteomics as a Tool in Revealing the Biology of Thyroid Eye Disease

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Background
Graves' ophthalmopathy (GO) is the most prevalent extra-thyroidal manifestation of Graves' disease (GD), an autoimmune disease. Published evidence suggest that orbital fibroblasts (OF) play a major role in GO. Autoantibodies target OFs, up-regulating cytokines and chemokines, which promote an inflammatory environment in the orbit. This induces overexpression of extra-cellular matrix proteins and accumulation of orbital fat which lead to an increase in orbital tissue volume (ophthalmopathy). Here, we investigate the proteomes of both orbital fat and fibroblasts to provide insight into the underlying biology of GO.

Methods
Orbital fat was obtained from patients with GO (n=5) and a normal group undergoing blepharoplasty (n=5). Fibroblasts grown from excised fat tissue and orbital fat samples were prepared for mass spectrometry using cell lysis buffers. Protein isolates were tryptically digested before being quantified using iTRAQ-based LC-MS/MS analysis (Tempo™ nano-MDLC system coupled to a QSTAR® Elite Hybrid LC-MS/MS). Peak-list generation, protein identification and quantification were accomplished with Protein-Pilot (v2.0.1, Applied Biosystems). Proteome datasets obtained were analysed using Ingenuity® Pathway Analysis.

Results
Proteomic analysis of OFs and fat resulted in 1937 and 226 hits respectively. Within the GO-Normal fibroblast pairs, 221 proteins were up-regulated and 223 down-regulated. The GO-Normal pairs derived from orbital fat had 24 up-regulated and 19 down-regulated proteins. Several pathways, most notably immunological and connective tissue disorders, were observed to be up-regulated.

Conclusion
The advent of modern MS technology over the past decade has helped refine proteomic analysis strategies; providing a robust platform for the identification of new diagnostic and prognostic targets. Several of the proteins identified in our study are currently being examined as possible key molecules in GO pathogenesis.

Keywords
Graves' Ophthalmopathy, Fibroblasts, Orbital Fat, Tandem Mass Spectrometry

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The role of cardiac metabolism perturbation in radiation-induced human heart disease : A proteomics analysis

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Background:
Epidemiological studies show a significant increase in ischemic heart disease (IHD) incidence associated with total external gamma-ray dose among Mayak plutonium enrichment plant workers. Our previous studies using mouse models suggest that persistent alteration of heart metabolism due to the inhibition of peroxisome proliferator-activated receptor (PPAR) alpha accompanies cardiac damage after high doses of ionising radiation. The aim of the present study was to elucidate the mechanism of radiation-induced IHD in humans.
Methods:
The cardiac proteome response to irradiation was analysed in Mayak workers who were exposed only to external doses of gamma rays. All participants were diagnosed during their lifetime with IHD that also was the cause of death. Label-free quantitative proteomics analysis was performed on tissue samples from the cardiac left ventricles of individuals stratified into four radiation dose groups (0 Gy, <100 mGy, 100 - 500 mGy, and >500 mGy).
Results:
The groups could be separated using principal component analysis based on all proteomics features. Proteome profiling showed a dose-dependent increase in the number of downregulated mitochondrial and structural proteins. Both proteomics and immunoblotting showed decreased expression of several oxidative stress responsive proteins in the irradiated hearts. The phosphorylation of transcription factor PPAR alpha was increased in a dose-dependent manner, which is indicative of a reduction in transcriptional activity with increased radiation dose.
Conclusion:
These data suggest that chronic external radiation enhances the risk for IHD by inhibiting PPAR alpha and altering the expression of mitochondrial, structural, and antioxidant components of the heart. Improving the function of PPAR alpha may serve as a useful preventive tool in radiation-induced IHD.

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A novel approach for tracing 15N tracer in proteins

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Background
Organic mass spectrometers, unlike isotope ratio mass spectrometers, are not designed for the accurate and precise measurements of isotopologue ratios as required in tracer studies. High resolution organic mass spectrometers are usually designed to maximize sensitivity and resolution of ion signals.

Methods
A novel method using UHPLC-QTOF-MS for the measurement of the incorporation of 15N nitrogen tracer has been developed. The method has been validated using recombinant ferritin obtained from cell cultures (E. coli) supplied with different percentages of 15N tracer. Ferritin extracts were collected via HPLC fractionation after cell lysis and subjected to trypsin digestion prior to UHPLC-QTOF-MS analysis. Four ferritin peptides (GDALYAMELALSLEK, ISEYVAQLR, NVPSEFEHVEK, VVLHPIK) were analyzed. The extent of 15N incorporation for the various cultures and replicates was determined using a newly developed algorithm and software.

Results
Calculated and gravimetrically determined percentages of 15N incorporation into the target peptides and, thus, the analyzed protein, showed a near perfect correlation. The correlation coefficients, R2, for the peptide plots were ≥ 0.9997 for all peptides. The developed technique permitted the determination of 15N incorporation into individual peptides at unprecedented repeatability with coefficients of variation ranging from 0% to 20.8 % for extreme tracer to tracee ratios and 0.19% to 2.86% for the rest of the ratios.

Conclusions
The conducted experiments have demonstrated that UHPLC Q-TOF-MS in combination with advanced algorithms for data analysis has the potential to deliver isotopic information in tracer studies at an accuracy and precision that approaches that of dedicated isotope ratio mass spectrometers (IRMS).

Keywords
15N tracer, QTOF-MS, Ferritin

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Plasma protein targets for early detection and monitoring of sinusoidal obstruction syndrome

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Background

Sinusoidal obstruction syndrome (SOS) is a serious complication that may occur in patients after hematopoietic stem cell transplantation (HSCT) as a result of hepatic and endothelial cell damage. The aim of this work was to search for SOS-associated plasma proteome changes potentially useful for early detection and monitoring of SOS complicatons in HSCT patients.

Methods

In total, 23 HSCT patients were involved in this study and they were divided into two cohorts: 12 HSCT patients with SOS manifestation, and 11 HSCT patients without complications that served as a control group. Plasma samples (isolated from the whole blood) of two different time points preceding the manifestation of SOS and two time points that followed were used; four different time points were used for a control group. Plasma proteins were separated using 2D SDS-PAGE and proteomes investigated and compared using Progenesis SameSpots software; proteins were identified using mass spectrometry.

Results

Comparing the protein expression profiles using principal component analysis showed that the samples were clearly separated into two distinct subgroups corresponding to patients with SOS and to those without complications. Further, 2D proteome map patterns were compared between SOS and control HSCT patients, and among all individuals from both the groups – alterated protein spots were selected for subsequent protein identification; so far, 103 different spots were selected. The preliminary results of protein identifications include proteins related to complement activation, assembly of protein complexes, cell adhesion, enzyme inhibition, innate imunne response, etc.

Conclusions

The preliminary results of this work identified potential plasma protein targets for SOS early detection or monitoring.

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Keywords

SOS; sinusoidal obstruction syndrome; hematopoietic stem cell transplantation; plasma proteome

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Proteome changes of extracellular vesicles isolated from the plasma of myelodysplastic syndrome patients

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Background

Myelodysplastic syndrome (MDS) encompasses a diverse range of oncohematological diseases characterized by ineffective hematopoiesis, blood cytopenias, and a progression to acute myeloid leukemia. Extracellular vesicles (exosomes, microvesicles and apoptotic bodies) are small membrane vesicles released to bodily fluids from majority, if not all, cell types and also cancer cells. These vesicles contain a broad repertoire of cargoes including proteins, nucleic acids and lipids. It is thought that extracellular vesicles play a key role in intercellular communication by transporting their content from one cell to another. Dysregulated pathways of cellular communication probably contribute to processes which lead to cancer development and progression. Recently, proteomic studies revealed differences between the protein content of normal and disease derived extracellular vesicles and it has been suggested that circulating vesicles can be a source of specific and hopefully reliable diagnostic markers.

Methods

In this study, 48 samples (12 RA-RARS, 12 RCMD, 12 RAEB, 12 healthy donors) were analyzed. Exosome-rich fraction of extracellular vesicles was isolated from defibrinated plasma using modified protocol for ExoQuick™ precipitation reagent. Dynamic light scattering was used to verify the presence of vesicles in the isolated fraction; 10 % of plasma protein contamination was observed. Exosomal proteins were separated by 2D SDS-PAGE (pI 4–7) and silverstained. The proteomes were compared and statistically processed with Progenesis SameSpots software.

Results

By comparing all four cohorts, 75 significantly ($p<0.05$, ANOVA) differing spots were found. Principal component analysis considering the significantly differing spots showed separation of the samples into three distinct groups (control samples, high-risk MDS, low-risk MDS). At the present, the proteins are being identified by nanoLC-MS/MS.

Conclusions

Results of this study could reveal the potential benefits of extracellular vesicles (exosomes) for myelodysplatic syndrome monitoring.

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Keywords

exosome, myelodysplastic syndrome, 2D SDS-PAGE

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MS-based epigenetic profiling of breast cancer samples for patient stratification and discovery of novel targets

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Background
Triple-negative breast cancers (TNBCs) comprise a highly diverse group of tumors, for which well-defined molecular targets have not yet been identified and targeted therapies do not exist. A better understanding of the molecular mechanisms underlying this type of disease is therefore needed in order to develop effective targeted therapies as well as discover biomarkers to be used as prognostic and predictive tools. Building on the increasing evidence that links aberrations in histone post-translational modifications (hPTMs) and cancer, in this study we employed a quantitative MS-based analysis of hPTMs to profile epigenetic patterns in different breast cancer subtypes, focusing on TNBCs in particular.

Methods
We profiled breast cancer patient samples using our recently developed PAT-H-MS (pathology tissue analysis of histones by mass spectrometry) method, which allows the MS-analysis of hPTM patterns from formalin fixed paraffin embedded tissues, in combination with a histone-focused super-SILAC approach. MS data were acquired on a high-resolution HF Q Exactive instrument.

Results
The MS-analysis of >100 breast cancer patient samples revealed epigenetic signatures, which included the H3 K27me3 and H3K9me3 marks, that distinguish different subtypes (Luminal A- and B-like, Her positive and TNBCs). Furthermore, we defined TNBC subgroups characterized by different epigenetic marks, as well as potential prognostic hPTM marks that differentiate TNBC patients with and without relapse after chemotherapy. HPTMs profiles were then intersected with global proteomics and RNA-seq patient data, identifying aberrantly expressed histone-modifying enzymes, which may serve as targets for epigenetic therapy for different breast cancer subtypes or TNBC subgroups.

Conclusions
The hPTM marks and signatures revealed by our work offer insights into epigenetic mechanisms that underlie breast cancer, and particularly TNBC, not only providing biomarkers useful for patient stratification, but also suggesting novel epigenetic pathways targetable for therapy.

Keywords
Quantiative MS, histone post-translational modifications, epigenetics, breast cancer

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Sirtuin 5 is probably a potential functional regulator of amino acid metabolism

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Background
Sirtuin 5 (SIRT5), localized in mitochondrial matrix, has been considered as a modulator of mitochondria that contributes to various kinds of diseases including neurological diseases. However, the underlying mechanism is unknown. Recent study revealed reliable interactions between SIRT5 and certain enzymes involved in amino acid metabolism. Investigating the potential regulation of SIRT5 on amino acid metabolism may provide basis for clinical treatment.

Methods
Tissues including colons, spleens, brains, thymuses, hearts, lungs, livers and kidneys were harvested from wild-type (WT) and Sirt5 knock-out (KO) mice. After sonication, extraction by ice cold 80% methanol, freeze-dry and resolution, the levels of amino acids in extracted metabolites were analyzed by LC-MS.

Results
Compared to WT mice, KO mice displayed different amino acid profiles of colons, spleens and especially brains, where there are 8 amino acids (Asp, Glu, Arg, Gln, Ala, Gly, Ser and Thr) showing decreased levels. Whereas, amino acid profiles displayed no significant differences in thymuses, hearts, lungs, livers and kidneys. Among amino acids whose levels changed, Glu, Gln, Ala and Gly are all significantly decreased in colons, spleens and brains.

Conclusions
KO mice exhibited different amino acid metabolism compared with WT mice, which led to conclusion that SIRT5 had a potential functional regulation on amino acid metabolism. Known that SIRT5 and some amino acid metabolic enzymes have reliable interactions, we speculate that SIRT5 could regulate related enzyme activity and subsequently influence amico acid metabolism, especially in brains.

Key words: SIRT5, amino acid profile, amino acid metabolism

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Radiation-induced endothelial inflammation is transferred via secretome to recipient cells in a STAT-driven process

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Background
It has been shown that women undergoing radiotherapy for left-sided breast cancer have a higher risk for developing cardiovascular disease later in life than those with right-sided breast cancer, probably due to the radiation damage of cardiac endothelium. The objective of this study was to elucidate proteins that are involved in the radiation response of human coronary artery endothelial cell line (HCAEC). Thereby, the focus was set on the analysis of the mechanism of communication to non-irradiated cells via the secretome.
Methods
Label-free proteomic analysis was performed on the cells and the surrounding medium 14 days post-irradiation using 10 Gy X-ray. For the secretome analysis, the medium of the irradiated cells was changed on day 13 to serum-free medium and was isolated 24 h after the medium change. The non-irradiated bystander cells were grown in this medium for 24 h before harvesting, lysis and label-free proteomic analysis.
Results
Irradiated cells showed an inflammatory response characterized by interferon gamma-related proteins modulated by STAT-proteins. Shared proteins between irradiated cells and the secretome revealed a transfer of radiation-induced inflammation into the medium by cytokines such as IL-6 and IL-8. In non-irradiated bystander cells a similar protein pattern was expressed as found in the irradiated cells and the secretome which can be allocated to the type I Interferon-signaling.
Conclusions
Irradiation of endothelial cells resulted in chronic inflammation and premature senescence. STAT3 played a key role in the induction of the inflammatory state inducing the expression of several interferon-related proteins. The secreted cytokine IL-6 is an important inducer of inflammation in the recipient cells. Those responded quickly (24 h) to the signal expressing a cluster of type I interferon-related proteins. These results are relevant in the study of radiation-induced cardiovascular disease.
Keywords
Irradiation, Inflammation, Bystander effect, endothelial cells

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insSTAGE-diging: the in solution upgrade of STAGE-diging protocol for proteomic sample preparation

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Background
In the avenue of Mass Spectrometry playing a key role in all aspects of cellular biology, instrumentation has undergone a remarkable evolution with significant gain in analysis speed, precision, consistency and sensitivity. Consequently, the quality of the samples to be analyzed, the reproducibility and robustness of sample preparation methods have now become major factors to determine the success of proteomics approaches. In practice, a good compromise - maximizing the effort/gain balance - needs to be found according to the aim of the specific experiment planned. Last year our group published a novel method for in-gel digestion, called STAGE-diging in which we speeded up sample preparation maintaining all benefits of standard in gel digestion.

Methods
Here we present a new protocol, named insSTAGE-diging, which can be applied to samples processed in solution. Briefly, an in solution approach in urea buffer is performed in a stage-tip, but the sample is also trapped in a polyacrylamide-gel matrix before digestion. This approach was compared with the well-established procedures for in solution sample preparation and with other techniques like FASP, Gel-assisted and in-Stage Tip. To avoid biological and technical variability, the same sample was processed in parallel with all the approaches, with and without detergents.

Results
insSTAGE-diging protocol is as effective and reproducible as other methods tested in terms of proteins identified and quantified. Moreover, it allows removal of detergent without several washing steps and it does not require any commercial device, with the exception of an home-made stage-tip, in common with all the techniques compared.

Conclusions
insSTAGE-diging protocol is easy to use, reduces sample handling and time consumption. Overall, it facilitates a good proteome characterization and can be applied in high-throughput projects with reasonable benefits compared with proteomics protocols in use up to now.

Keywords: Mass Spectrometry, sample preparation, STAGE-diging, in solution digestion

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Fast Phosphoproteomics Analysis of Human Jurkat T cells by HIFU-TiO2-SCX-LC-MS/MS

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Background: To date, substantial efforts have been directed toward identification and characterization of the maximum number of phosphorylation sites per experiment. Thus, a new strategy for the Fast Phosphoproteomics Analysis is presented in this work.

Methods: The performance of the method was established for the global phosphoproteome analysis of un-stimulated human Jurkat leukemia T cells (E6.1). The proposed method is based on six main steps: (a) cell lysis and protein extraction (time: 45 min), (b) in-solution trypsin digestion accelerated under an ultrasonic field provided by high-intensity focused ultrasound (HIFU) (time: 10 min), (c) a single step of phosphopeptide enrichment using TiO_2 (time: 90 min), (d) fractionation by strong cation exchange chromatography (SCX) (time: 60 min), (e) analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a high-resolution LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) (time: 60 min/run), and (f) data analysis using BYONIC and SEQUEST-HT (Proteome Discoverer 2.1, Thermo Fisher Scientific) (time: 60 min).

Results: Using this accelerated workflow, 15,367 phosphorylation sites from 13,029 different phosphopeptides belonging to 3,163 different phosphoproteins were efficiently identified with high-throughput and reproducibility in less than 15h.

Conclusions: Results demonstrates that the present strategy, HIFU-TiO_2-SCX-LC-MS/MS, is the fastest analytical method reported to date for generating reproducible large-scale phosphoproteomics datasets in a limited time (<15h).

Keywords: Phosphoproteomics, high-intensity focused ultrasound (HIFU), TiO_2, strong cation exchange chromatography (SCX), mass spectrometry, human Jurkat T cells

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Geena2 - a public tool for the automation of MALDI/ToF spectra pre-processing

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Background
Automation of bioinformatics analysis is essential for the elaboration of data from high-throughput technologies, including proteomics. It also supports replicability and reproducibility of experiments. Little is available for scientists which do not have a strong computer science background. We have developed Geena2, a public tool for the automation of MALDI/ToF spectra pre-processing.
Methods
Geena2 implements: a) unification of isotopic abundances for the same molecule, b) normalization of data against a standard, c) background noise reduction, d) computation of an average spectrum representative for replicate spectra, e) alignment of average spectra. Its input consists in lists of peaks. Various parameters are available to cope with the user needs. The output includes the average spectra and their alignment. All intermediate results may be downloaded.
Results
Geena2 was first used for the evaluation of the effects of long-term cryopreservation on serum samples: the abundance of several signals, mainly peptides related to blood coagulation and to complement system activation, changes significantly during cryopreservation, even at -80°C. It was then used in two published retrospective studies on the correlation between serum peptidomic profiles and cancer. In the first, we found out a correlation between C3f serum level and risk to develop a breast cancer in patients affected by gross cystic disease of the breast. The second demonstrated a higher risk of relapse in patients having high serum level of angiotensin II among women who undergone surgery for a breast cancer.
Conclusions
Geena2 has shown its ability to automate many steps in the pre-processing of MALDI/ToF spectra (http://bioinformatics.hsanmartino.it/geena2/). In conjunction with some powerful statistical tool, it may constitute a simple and effective tool for scientists with little computer expertise. GeenaR, a new implementation able to exploit the power of R modules, is under development.
Keywords
MALDI/ToF, web server, automated data preprocessing

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Use of MALDI imaging technology to predict the response to antiangiogenic therapy in colorectal cancer

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Background: Angiogenesis, the process by which new blood vessels are produced from pre-existing ones, is a key step in the tumor progression and metastatic spread of tumor cells. Although antiangiogenic therapy has demonstrated its efficacy, not all patients respond in the same way and biomarkers that distinguish between groups of responders and non-responders patients are needed. More specifically there are a number of long term responders with an extended period of treatment before progression of disease. Mass spectrometry imaging (MSI) is a very promising analytical technique in oncology and particularly in the discovery of biomarkers that predict treatment response.
Methods: We used 29 formalin-fixed paraffin-embedded tumor tissues from 22 patients with metastatic colorectal cancer to predict the response to the antiangiogenic drug bevacizumab based on the proteomic information obtained with matrix-assisted laser desorption/ionization (MALDI) MSI. First, starting only from proteomic hyper-spectral data, spatially-aware shrunken centroid unsupervised analysis was used to virtually micro-dissect tumor tissue away from whole specimen. Second, based on Random Forest Algorithm a classification was realized according to time to progression. Patients were classified into two groups corresponding to less than twelve months of treatment or equal or more than twelve months of treatment. Both methodologies were performed under R environment (Cardinal and Caret packages respectively).
Results: We were able to precisely distinguish tumor from healthy tissue and to virtually dissect the former for subsequent classification analysis. The best classifier generated from this cohort of patients showed an AUC of 0.83 (specificity 0.760, sensitivity 0.745), as determined by cross-validation.
Conclusions: This study highlights the usefulness of MALDI-MSI both for the dissection of tumor regions without any other device and the prediction of response to antiangiogenic treatment. A validation test will confirm these preliminary results.
Key words: MALDI-MSI, antiangiogenic therapy, bevacizumab, Random Forest algorithm, spatially-aware segmentation.

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Role of lipolysis in cancer

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Metabolic rewiring is a dominant factor within tumor initiation and progression. Defining the pathways that are limiting for cancer progression can be exploited as therapeutic targets. Fatty acid metabolism is a key process in initiation of metastasis (via import of external fatty acids) and also in cancer cell proliferation (via de novo fatty acid synthesis). Lipases (responsible for mobilisation of fatty acids from lipids) play a central role in providing fatty acids and oncogenic signaling lipid molecules thereby affecting cancer aggressiveness. The rate limiting lipase in intracellular triacylglycerol mobilisation Adipose Triglyceride Lipase (ATGL) has been found to be down regulated in many human tumors and its depletion induces mouse pulmonary neoplasia. Consinstently, we report here that CRISPR/Cas9 deletion of ATGL in A549 lung adenocarcinoma cells leads to increased intracellular lipid accumulation and in addition increases cell proliferation and cell mobility. Our label free quantitative proteomics and transcriptomics data show that protein and mRNA expression levels of the proto-oncogene Src and enzymes of one-carbon metabolism are significantly altered. Increased cell motility of ATGL knock out A549 lung adenocarcinoma cells is reversed by pharmacologic inhibition of Src. One-carbon metabolism is a central biosynthetic hub of cellular proliferation producing building blocks for DNA synthesis, providing methylation equivalents for transcriptional regulation and is a major source for cellular NADPH, a biosynthetic bottleneck in rapidly proliferating cells required for e.g. de novo fatty acid synthesis and oxidative stress response systems. We currently perform metabolomics analyses to better understand the complex interplay of fatty acid metabolism with central carbon and one carbon metabolism to drive cell proliferation directly on the metabolite level.

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Developement of site-specific glycome analysis method of complex glycoproteome

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Background: Until recently, “glycoproteome analysis” refers to the large-scale identification of glycopeptides after removal of glycan moieties from glycopeptides, e.g., using PNGase. This approach provides a large numbers of glycosylation sites for glycosylated proteins; however, glycan structure information on each site cannot be clarified. To elucidate the role of glycans, it is important to reveal what glycans are attached to which sites and how glycans change in association with diseases and stimuli. Therefore, we attempted to develop a method to assign site-specific glycomes on the proteome scale.
Methods: The approach of our method is as follows: 1) An aliquot of enriched glycopeptides is analyzed by LC/MS using a high accuracy mass spectrometer, 2) Glycopeptide signals are assigned by non-MS/MS-dependent manner to obtain their accurate masses and retention times, 3) A glycopeptide core sequence list is prepared by conventional LC/MS/MS analysis of de-glycosylated peptides, and 4) By comparing mass of core peptide and glycopeptide, combinations of core peptide and glycan composition are predicted. To improve prediction accuracy, we examined criteria for selecting the most likely combination.
Results: We applied this method to the analysis of glycopeptides prepared from mouse tissues, and obtained large-scale site-specific glycome information. To evaluate the method, the prediction results were compared with the results obtained by data-dependent HCD-MS/MS analysis of glycopeptides. Based on the comparison, the criteria for selecting the most likely prediction were optimized.
Conclusions: We were able to establish a method for large-scale site-specific glycome analysis. This method has a higher sensitivity than MS/MS-dependent manners, and it is possible to obtain a wide range of site-specific glycome information. Therefore, this method will contribute to revealing the mechanism of generating site-specific glycan heterogeneity and to developing disease glycobiomarkers.

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Regulation of Peroxisomal Matrix Protein Import by Peroxin Phosphorylation

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Background
Peroxisomes are ubiquitous, single membrane-bound organelles that fulfill essential metabolic functions. Dysfunctions in peroxisome biogenesis or metabolism cause severe, often lethal diseases in humans, e.g. the Zellweger Syndrome, underscoring the importance of this organelle for life. Peroxisomal matrix proteins are nucleus-encoded and posttranslationally imported through distinct transient import pores. In Saccharomyces cerevisiae, the cytosolic receptor for matrix proteins carrying a peroxisomal targeting sequence (PTS) 1, Pex5p, and the membrane protein Pex14p form the PTS1 pore. Functional import further relies on a ubiquitination-dependent (re)cycling of Pex5p. In this work, we studied the role of Pex5p phosphorylation for the import of PTS1 proteins.

Methods
We used high-resolution LC-MS combined with phosphopeptide enrichment by TiO2 to map and quantify phosphosites of affinity-purified endogenous Pex5p from yeast. In vitro kinase assays using radiolabeled ATP and autoradiography as well as non-labeled ATP and LC-MS providing site-specific information about phosphosites were used to identify kinases mediating Pex5p phosphorylation. Pex5p phosphosite mutants were generated and analyzed for import defects using fluorescence microscopy and biochemical assays. We further studied cross-talk between Pex5p phosphorylation and ubiquitination using Pex5p mutants in which ubiquitination is prevented.

Results
We show that Pex5p is multiply phosphorylated in vivo and targeted by several cytosolic kinases including members of the casein kinase family. MS-based quantification enabled the determination of preferred kinase substrate sites. A Pex5p mutant with serine-to-aspartate exchange in the cargo-binding region exhibits a partial import defect for peroxisomal matrix proteins. Biochemical data further provide evidence for a cross-talk between Pex5p phosphorylation and ubiquitination.

Conclusion
We here present the so far most comprehensive phosphorylation map of Pex5p including information of kinase-substrate relationships. Results of our functional analyses point to a functional role of Pex5p phosphorylation for peroxisomal matrix protein import.

Keywords
Phosphoproteomics, Yeast, Peroxisomal matrix protein import, Pex5p phosphorylation, Kinase-substrate relationships

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To deplete or to equalize? Two pre-analytical strategies for human cerebrospinal fluid analysis.

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Background
Human cerebrospinal fluid (CSF) is a potential source of biomarkers for neurological diseases. Similarly to blood plasma, proteomic analysis of CSF is complicated by presence of several highly abundant proteins obscuring the less abundant proteins and limiting the proteome coverage. Unfortunately, there is no established method for dealing with the highly abundant CSF proteins .
Methods
Applicability of two depletion strategies developed for human plasma was tested in human CSF samples. We compared an immunodepletion method using MARS 14 column (Agilent) side-by-side with an alternative “equalizing” strategy using hexapeptide ligand library (ProteoMiner, Bio-Rad).
Results
LC-MS/MS analysis of raw (non-depleted) human CSF identified only 400 proteins while MARS 14-immunodepleted CSF sample provided 740 identified proteins. CSF sample “equalized” with ProteoMiner provided roughly 600 identifications. We also analyzed the “waste” fractions (proteins retained by MARS 14 column and the “flow through” fraction from the ProteoMiner beads). The MARS column retained almost 180 proteins including 90 proteins not found in the depleted CSF sample. Similarly, the Proteominer “flow through” fraction contained 280 proteins, including 80 not observed in the “equalized” CSF sample. Combined results from crude CSF and the two fractions obtained by MARS 14 (non-treated CSF + depleted CSF + column-retained proteins) provided 880 unique protein identifications. Combined results for ProteoMiner ligand library method contained 740 unique protein identifications.
Conclusions
Both methods significantly improved CSF proteome coverage. MARS 14 immunodepletion strategy was more efficient, increasing proteome coverage roughly 1.85-fold compared to non-depleted CSF. To maximize the CSF proteome coverage, it is beneficial to analyze also the fraction of proteins retained by the column and, eventually, also the original unfractionated CSF sample. Such a combined triple analysis increases CSF proteome coverage by a factor 1.2 (880 IDs) compared to depleted CSF only (740 IDs).
Keywords
Cerebrospinal fluid, CSF, abundant proteins, depletion

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Robust High Throughput DIA Plasma Proteomics Pipeline Finds Body Mass Index-associated Increases in Inflammatory Pathways

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Background:
Plasma is the most widely-used body fluid for the discovery of biomarkers. Despite its well defined composition, large numbers of samples have to be analyzed to identify meaningful biomarker candidates. To address this need, we developed a high-throughput plasma proteomics pipeline using 96-well plates, DIA and capillary-flow LC that allows for the complete processing and analysis of 96 samples within one work week using one mass spectrometer.

Methods:
Plasma samples from 62 patients were individually trypsinized and desalted using 96-well PVDF membrane and C18 plates. For the spectral library generation, plasma from 62 participants was pooled and trypsinized with and without i) prior depletion of the Top14 abundant proteins and ii) subsequent high-pH fractionation. The different digests were analyzed on a Q Exactive MS using either DDA or DIA with a capillary-flow LC. The data was analyzed with MaxQuant and Spectronaut.

Results:
For evaluating the performance of our DIA plasma proteomics pipeline, we analyzed a pooled plasma sample in triplicates, identifying on average 467±2 proteins with 82.5% overlap, i.e. twice the number of proteins identified using comparable DDA workflows. Using <1 µl of plasma, and <1 hour of instrument time, our high-throughput plasma proteome maps cover >5 orders of magnitude dynamic range.
We next analyzed 62 plasma samples identifying in total 664 proteins, and on average 433±36.5 plasma proteins/sample. Detecting the expected sex-dependent changes in the plasma proteomes validated our pipeline. Correlating BMIs of the patients with changes in the plasma proteomes identified 18 (23) (anti-)correlating proteins. Intriguingly, the list of positively correlating proteins was enriched in inflammatory proteins, demonstrating an association between inflammatory processes and obesity.

Conclusion:
Our plasma proteomics pipeline is robust, easily automatable, and applicable to small (e.g. fingerstick) plasma sample volumes. Using the pipeline, we demonstrate an association between increased BMI and inflammatory proteins.

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Effects of amino acid supplementation on the performance of gilthead seabream using 2D-DIGE

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Background: Human fish consumption is continuously growing and aquaculture production costs need to be minimized, while keeping optimal growth and fish health. Plant-based protein ingredients are an alternative for fish diets but may influence growth and stress due to imbalanced amino acids profiles. Objectives: evaluation of the functional effect of diet supplementation with three amino acids. Histidine: involved in biochemical changes which occur during inflammation; threonine: involved in the immune system response, and tryptophan: involved in stress and inflammatory responses. Differential proteomics will show the immune status of gilthead seabream.
Methods: Gilthead seabream were fed with three diets to evaluate the effect of amino acids supplementation on fish performance. Diets were formulated based on estimated requirements. Diet 1 had 0% of the three amino acids, diet 2 with 1.1% (w/w) of supplementation and diet 3 with 1.5% (w/w). Fish were fed twice a day, ad libitum and maintained in natural conditions. After 2 weeks fish were lethally anesthetized, liver was collected and kept at -80ºC for further analyses. Proteins were extracted using a DIGE buffer and quantified by the Bradford method. Proteins were separated using 2D-DIGE on 24 cm strips with pH 4-7. Differentially expressed proteins were identified using mass spectrometry.
Results: Gels were analyzed using SameSpots software, 41 spots showed significant difference (one-way ANOVA, $p < 0.05$) between the diets. Identified proteins were related to the stress response – namely hyperosmotic glycine rich protein – and energy metabolism – namely enolase 1 and heart-type fatty acid binding protein.
Conclusions: As the mechanisms of stress and energy were triggered in fish fed diet 1 (no supplementation of the amino acids) we might conclude that even without the amino acids in the diet, fish were able to overexpress some specific proteins for their performance.
Keywords: Proteomics, Amino acids, Gilthead seabream, Aquaculture

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Identification of serum proteins that can delineate acute Hantavirus infection

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Background: Seoul hantavirus has become endemic in the UK and mainly infects farmers and pet rat owners. This is associated with acute renal failure. We have investigated the serum proteome to identify putative markers of hantavirus infection. In this study, we focus on one individual who had an acute infection during pregnancy. Uniquely a longitudinal analysis of infection and the effects on the serum proteome was performed, making use of blood samples taken before infection. These results were compared with single samples from other patients with and without acute hantavirus infection. This is the first time proteomics has been applied to an analysis of the serum proteome during a pregnancy complicated by Hantavirus infection.

Methods: Abundant serum proteins were depleted from samples. Label free proteomics was used to identify and quantify proteins in the experimental and control patient groups. Progenesis software was used for protein quantification. GProX software was used to cluster proteins identified in the serum of the pregnant individual into distinct groups reflecting temporal regulation. ELISA was used to provide an independent validation of selected proteome changes in a separate patient cohort.

Results: Several proteins were differentially abundant in both the pregnant individual and in other patients with acute hantavirus infection. One protein increased in abundance, galectin-3-binding protein, has previously been associated with Puumala hantavirus infection and was also confirmed by ELISA with a larger patient cohort. The data indicated that the acute phase response and coagulation pathways were activated in hantavirus infection.

Conclusions: Quantitative proteomics can be used to investigate and distinguish changes in the serum proteome during acute Seoul hantavirus infection. This work is being extended to identify potential serum biomarkers for diagnosis of the acute phase of disease and the biology underlying the infection and host response.

Keywords: Hantavirus, Seoul virus, renal failure, proteomics

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The application of Commensal Computing for massively parallel analysis of proteomics data

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Background:
Post translational modifications (PTMs) to proteins significantly alters their function. PTMs can be studied using mass spectrometry (MS), however they are challenging to identify using “blind searching” methods from non-PTM enriched MS data sets. Such searches either restrict the database size or require large amounts of CPU time. We are pioneering a new method - termed ‘commensal computing’. Here the full analysis is performed using 1000s of threads, as a browser embedded application. For each parallel thread, a little client-side CPU time is ‘donated’ to the task, users are aware that their idle time is being used but do not notice any degradation in performance.

Methods:
The tool uses a mix of server and client side technologies. The server splits the task into small work units provided to the client as packages containing a spectrum, candidate peptide sequences and the possible PTMs that could have produced the spectrum. These are analysed using an asynchronous JavaScript method that returns a list of scored peptide-spectrum matches (PSMs) for each candidate spectrum analysed. The server re-assembles all the scored PSMs for global statistical analysis and presents the results back to the user.

Results:
The tool is available for proteomics labs to upload data sets for blind modification searches. We have also commenced the re-analysis of all publically available human proteome data sets, the final results made available for all those interested in the PTM capacity of the human proteome.

Conclusions:
Distributed processing of this type of task has been proven to be both possible and desirable, as an alternative to the use of expensive high-performance computing clusters.

Keywords
“blind PTM searching”, “distributed computing”, “bioinformatics”, “protein identification”

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MRM of synucleins in CSF reveals alterations in Alzheimer´s and Creutzfeldt-Jakob disease but not synucleinopathies

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Background
α-Synuclein (αSyn) is a major constituent of proteinaceous aggregates in neurodegenerative diseases such as Parkinson´s disease (PD) and a potential biomarker candidate. However, studies about αSyn in cerebrospinal fluid (CSF) in diseases are inconsistent and mainly based on immunological assays. Quantitative information about β-synuclein (βSyn) and γ-synuclein (γSyn) in CSF is not available.

Methods
We used LC-MRM for the simultaneous quantification of αSyn, βSyn, γSyn and hemoglobin in CSF with high sequence coverage (70%) of αSyn to validate previous, ELISA-based results and characterize synucleins in CSF in more detail.

Results
The MRM has high sensitivity in the low pg/mL range (3-30pg/mL full-length αSyn) using 200µL CSF. A high portion of CSF αSyn is present in the N-terminally acetylated form and the concentration of unmodified peptides in the non-amyloid component region is about 40% lower than in the N-terminal region. Synuclein concentrations show a high correlation with each other in CSF ($r>0.80$) and in contrast to αSyn and γSyn, βSyn is not affected by blood contamination. CSF αSyn, βSyn and γSyn concentrations were increased in Alzheimer´s and Creutzfeldt-Jakob disease but not altered in PD, PD dementia (PDD), Lewy body dementia and atypical parkinsonian syndromes. The ratio βSyn/αSyn was increased in PDD (1.49 ± 0.38, $p<0.05$) compared with PD (1.11 ± 0.26) and controls (1.15 ± 0.28). βSyn shows a high correlation with CSF tau concentrations ($r=0.86$, $p<0.0001$, $n=125$).

Conclusions
This is the first study quantifying synucleins in CSF by MRM and we could not confirm previous observations of reduced αSyn in PD. Our results indicate that CSF synuclein concentrations are rather general markers of synaptic degeneration than specific for synucleinopathies. βsyn is an attractive biomarker candidate that might be used as an alternative to or in combination with tau in AD and CJD diagnosis and in combination with αSyn it is a biomarker candidate for PDD.

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A fast and sensitive LC-MS method for quantitation of a novel prodrug against Wilson's disease

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Wilson's disease is a rare genetic disorder, due to copper homeostasis dysfunction, that affects approximatively 1 of 30,000 people worldwide. Under normal condition, the body needs a small amount of copper used as cofactor by many enzymes playing vital to stay in good physical shape. However, too much copper is poisonous. Normally, the liver filters extra copper and releases it into bile that carries toxins and wastes out of the body through the gastrointestinal tract. In WD, mutations of the ATP7B gene induces an impaired functioning of a Cu-ATPase, impaired Cu detoxification in the liver and copper overload in the liver, brain, eyes and other organs. Over time, the extra copper can lead to organ damage that may cause death. When WD is diagnosed early and treated effectively, people with the condition usually can have good. Nowadays, WD is treated life-long by systemic chelation therapy, which is not satisfactory. Therefore the design of selective and efficient treatments is of great interest. Recently, we have proposed a strategy based on bioinorganic chemistry to design novel drugs by taking inspiration from the biological copper cell transporters specific. The targeting is based on carbohydrates subunit that induce endocytosis in hepatocytes by the asialoglycoprotein receptor mainly expressed at the cell surface and plays a role in the clearance of desialylated proteins from the serum. To evaluate the potential of this new glycoconjugates-chelator as a potential therapeutic agent we have undertaken in vitro and whole animal studies to characterize its pharmacokinetics. A accurate and selective LC-SRM method was developed and validated for the quantification of this Chelator in serum, urine and liver tissue samples. The specificity, linearity, limit of quantitation, precision, accuracy, recoveries and stability were determined. Validated LC-SRM method was applied to a pharmacokinetic studyin which Cu-chelator was administered IV to mice.

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Impact of sulfation on SDF-1/receptor's extracellular domain interaction by affinity electrophoresis and mass spectrometry

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Background: Sulfation is one of the most important post-translational modifications of proteins. The known sulfated proteins are mostly cell receptors and among them, CXCR4 attracts growing attention because of its involvement in numerous physio-pathological processes (immune response, HIV infection). The extracellular domain of CXCR4, containing three tyrosine residues known to be sulfated, is important for the interaction with its specific ligand, the SDF-1/CXCL12 chemokine (Stromal cell-derived factor-1). The role of sulfation in this interaction remains to be established.

Methods: The 38 amino-acid peptide P38 corresponding to the extracellular domain of the CXCR4 receptor was chemically synthesized and sulfated on the three different tyrosine residues, 7, 12, and 21 respectively, and at different levels (mono-, di- or tri-sulfated). The impact of both sulfate distribution and position of sulfate groups on the interaction between P38 and SDF-1 is studied by affinity capillary electrophoresis (ACE) hyphenated to electrospray mass spectrometry (ESI-MS). ACE allows the detection of non-covalent complexes freely formed in solution and without partner immobilization and enables the determination of dissociation constants. The ACE-MS coupling in non-denaturing conditions allows the characterization of non-covalent complexes (stoichiometry).

Results: An interaction between P38 and SDF-1α was highlighted by ACE. It was strongly enhanced by the increase of P38 sulfation degree. Low dissociation constants (μM) were calculated. The complex stoichiometry was then determined using ACE on-line coupled to ESI-MS.

Conclusions: Sulfation seems to have an important role in the affinity between the SDF-1α chemokine and its receptor CXCR4. Higher is the P38 sulfation degree and stronger is the binding between the two partners. Other analytical tools will be considered such as hydroxyl-radical protein footprinting to determine the complex interaction sites.

Keywords: Sulfation, SDF-1/CXCR4 complex, affinity capillary electrophoresis, mass spectrometry

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Social network architecture of human immune cells unveiled by quantitative proteomics

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The immune system is unique in its dynamic interplay between numerous cell types. However, a system-wide view of how immune cells communicate to protect against disease has not been established. Here, we applied high-resolution mass spectrometry-based proteomics to characterize 28 primary human hematopoietic cell populations in steady and activated states at a depth of > 10,000 proteins in total. Protein copy numbers reveal a specialization of immune cells for ligand and receptor expression, thereby connecting distinct immune functions. By integrating total and secreted proteomes, we establish a 'social network' of communicating cells and discover fundamental structures of intercellular information exchange as well as novel connections between cell types. Our publicly accessible (www.immprot.org) proteomic resource provides a framework for the orchestration of cellular interplay and a reference for altered communication associated with pathology.

Keywords: Immune cell proteomes, Secretomes, Cell-cell communication network

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Search Engine Score Independent Re-Scoring of PSMs Using Predicted Ion Peak Intensities

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Background
Machine learning-based methods for re-scoring Peptide-Spectrum Matches (PSMs), such as Percolator [Käll 2007], have thoroughly demonstrated their usefulness. Percolator relies on features built from the search engine output, the score being one of the most relevant features. We explore the combination of this approach with another proteomics tool that uses machine learning - MS2PIP [Degroeve 2013], which is used to predict fragment ion peak intensities given a peptide's sequence, modifications and charge. We show that it is possible to replace Percolator's default features with the ones derived from the computationally predicted spectra, while maintaining the final number of PSMs, creating a re-scoring system independent from the search engine scores.

Methods
The pyrococcus furiosus standard for evaluation of peptide identification algorithms [Vaudel 2012] was used in this study. This data was searched with MSGF+ [Kim 2014] and a list of identified peptides was obtained, which was used by MS2PIP to predict a set of spectra. Several features were extracted from the comparison between the predicted spectra and the spectra to which the sequence was matched, such as the correlation, distribution of errors, etc. We then applied Percolator using the features compiled from each pair of spectra in place of the default ones, derived from the search engine scores.

Results
Using exclusively the features computed from the comparison between the predicted and assigned spectra yields slightly more PSMs at the same False Discovery Rate (FDR) than using the default features, with no increase of entrapment PSM hits.

Conclusions
The removal of the search engine scores from the Percolator features is of high relevance, as it allows for a re-scoring framework based entirely on the spectra and comparison to their computationally predicted pairs. This could easily be applied to results obtained with any search engine.

Keywords
scoring function, machine learning, peptide identification

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Role of adipocyte plasma membrane proteome in adipocyte differentiation and adipose function

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Background
Metabolic syndrome is a major public health burden worldwide and is a risk factor for cardiovascular disease. It affects around a quarter of the world's adult population, with a prevalence of 10-30% in the aging population of most European countries. Adipose tissue is a key regulator of metabolic homeostasis; however there is little information on how the adipose plasma membrane (PM) proteome, a key player in nutrient and drug transport and cell signalling, changes during metabolic disease. We have used an in vitro model to investigate how the adipocyte PM proteome changes during adipocyte differentiation and insulin stimulation.

Methods
Murine 3T3-F442A pre-adipocytes were differentiated into mature adipocytes over a period of 10 days. Mature adipocytes were serum starved overnight and stimulated with 200nM insulin for 20 minutes or left untreated (basal state). Colloidal silica bead isolation was used to separate plasma membrane proteins which were analysed by LC-MS/MS with hybrid quadrupole time-of-flight mass analyser (QTOF). Protein identification and pathway analysis were performed using ProteinPilot and Reactome Pathway Database respectively.

Results
This study identified 226 PM proteins in preadipocytes; 316 PM proteins in unstimulated differentiating adipocytes and 289 PM proteins in insulin-stimulated differentiating adipocytes. 228 PM proteins were differentially regulated during adipogenesis; we also identified 110 PM proteins differentially regulated by insulin stimulation. Important PM proteins differentially regulated during adipogenesis include RACK1 (10-fold upregulation) and NEDD4 (2-fold upregulation); insulin stimulation led to the upregulation of Caveolin-1 (1.75 fold) in differentiating adipocytes. Pathway analysis revealed that vesicle-mediated transport and membrane trafficking were overrepresented during adipogenesis and insulin stimulation.

Conclusions
We present a global analysis of the PM proteome during adipocyte differentiation and insulin stimulation. The role of known and novel PM proteins will be further validated using functional assays and in vitro human models.

Keywords: metabolic disease; adipocyte; plasma membrane proteome.

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In-depth proteome-profiling to evaluate a novel combinatory metronomic treatment for therapy-resistant multiple myeloma patients

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Background

Multiple myeloma is an incurable plasma cell tumor of the bone-marrow. Presently different therapeutic strategies exist to treat this disease; however, there are no stratification possibilities to determine the best therapy for each individual patient. Actually, there is a lack of screening methods to assess the status of the tumor cells and the tumor-associated microenvironment of each patient, which would be of enormous value. In the present work, a new therapeutic approach for therapy-resistant myeloma patients, based on a combinatory treatment using metronomic chemotherapy of tumor and stromal cells, was evaluated. A minimal-invasive method using serum samples for proteome profiling was chosen to assess mechanisms underlying the beneficial effects of this therapy and to provide information for possible therapeutic improvements.

Methods

Serum samples of seven patients participating in the clinical study were analyzed, taking samples before and at different time points after the beginning of the therapy. Samples of patients and of three healthy volunteers were depleted, proteins were digested with trypsin and resulting peptides were subjected to a nano-LC-MS/MS analysis using a QExactive orbitrap mass spectrometer for proteome profiling. Identification of proteins, label-free quantification and statistical analyses were performed using the MaxQuant software.

Results

A total of 381 protein groups was quantified across all samples. Multiple comparisons of individual patient profiles and control samples revealed possible treatment- and disease-associated marker molecules. A general regulation of proteins involved in hemostasis, angiogenesis, inflammation, acute phase reaction, proliferation, immunomodulation and tissue remodeling was determined. The protein regulation observed in response to the therapy can be correlated especially with functional changes in the tumor microenvironment.

Conclusions

Proteome profiling proved to be successful for determining possible prognostic markers, as well as markers which could monitor the therapy response of individual patients.

Keywords

Multiple Myeloma, tumor microenvironment, metronomic chemotherapy, serum proteomics

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Novel target identification in Alzheimer's disease brain by combined mass spectrometry imaging and subcellular proteomics

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Background: Understanding the neurobiology of Alzheimer's disease (AD) is of great importance in order to develop new medicines that can combat this progressive and debilitating disorder. We present a combination of subcellular proteomics by nUPLC-IMS-MS and MALDI-MSI that together provide a novel tool for identifying and localizing proteomic alterations in AD brain tissue.
Methods: Frozen human prefrontal cortex brain samples from four AD patients and four matched non-AD controls were prepared for DAB-immunohistochemistry, MALDI-MSI or nUPLC-IMS-MS analysis. A pathological characterization was performed by immunostaining tissue sections with 6E10 (mAb) and AT8 (mAb) followed by DAB reaction, respectively for targeting amyloid and tau pathology. Subsequent tissue sections were mounted to ITO-coated glass slides and introduced to on-tissue digestion and/or various matrix solutions using the ImagePrep robot. Data acquisitions were performed using the Ultraflextreme instrument followed by data analysis in SCiLS Lab software. Furthermore, larger tissue sections were prepared for nUPLC-IMS-MS analysis by using an in-house FASP protocol. Data acquisitions (shotgun approach) were performed on Bruker timsTOF instrument followed by identification and statistical analysis in MaxQuant and Perseus software.
Results: All methods confirmed AD pathology in all AD samples by showing presence of AD markers. Furthermore, ionization efficiency of Aβ was found to vary significantly depending on choice of matrix. MALDI-MSI and discovery proteomics revealed several discriminating proteins that varied significantly when comparing AD to the control. It was found that part of the identified proteins could be matched between the two methods when comparing the molecular weight of the target molecules.
Conclusion: MALDI-MSI and nUPLC-IMS-MS are useful tools for localizing and identifying discriminating proteins and PTMs in tissue samples from AD patients compared to control. However, in order to confirm the presence of detected targets additional histological staining is required.

Keywords: MALDI-MSI, nUPLC-IMS-MS, Ultraflextreme, tims-TOF, Alzheimer's disease.

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ProteomicsDB enables custom spectral library and transition list generation

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Background:
The first draft of the human proteome published in 2014 became possible by compiling many human proteome projects both from our own work as well as published data from other laboratories into a publically available database called ProteomicsDB. In addition to this, ProteomicsDB hosts more than 3 million spectra of more than 600.000 synthetic peptides covering the entire human proteome from the ProteomeTools project. By providing high quality peptide identifications in combination with their proteotypicity, both data sources can be used to build custom high quality spectral libraries enabling the guided development of targeted assays or the analysis of DIA experiments.

Methods:
Spectral libraries of all ProteomeTools experiments were built in Mascot and SpectraST. All experiments in ProteomicsDB were scaled to the same iRT range based on 40 internal RT peptides in all ProteomeTools run. Afterwards non linear regressions were applied to generate a proteome wide model to predict retention times for peptides in ProteomicsDB.

Results:
We present a new analytical feature of ProteomicsDB which allows the guided development of spectral libraries and transition lists for the development and analysis of DIA experiments by making us of the vast amounts of synthetic and experimental data. The retention time alignment allows to integrate most experimental data stored in ProteomicsDB and is used for the prediction of interfering transitions as well as retention time prediction. This, in combination with experimental proteotypicity values, allows a semi-automatic data-driven selection of high quality and reproducibly observed peptides for almost any protein of interest.

Conclusion:
ProteomicsDB offers three new analytical tools for the custom generation of transition lists and spectral libraries as well as the validation of external spectral libraries. A guided and fully configurable step-by-step wizard enables users to generate and export these in different formats.

Keywords:
database, bioinformatics, spectral library, targeted assays

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Large-scale proteomic analysis of SIRT1- and tissue-dependent acetylproteome in mouse liver, testis and muscle

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Background
SIRT1 (NAD-dependent protein deacetylase Sirtuin-1) regulates the expression of genes by deacetylating their transcription factors and co-regulators. These can be involved in a wide range of functions such as cell cycle, DNA damage, metabolism, aging and spermatogenesis. Sirt1 is differentially expressed in tissues and its activity can be regulated through post-translational modifications or expression of tissue-specific isoforms. Furthermore, SIRT1 activity depends on NAD+ levels, which is altered by starvation and metabolic changes associated with caloric restriction. In order to identify substrates of SIRT1 in vivo and to gain insights into tissue- and diet-dependent regulations by SIRT1, we analysed SIRT1-dependent acetylations in liver, muscle and testis of fed and starved mice.
Methods
Tissues were harvested from fed and starved wild-type control mice or mice with conditional Sirt1-KO. After lysis and trypsin digestion, acetylated peptides were enriched by immuno-precipitation using a specific antibody targeting acetylations before being injected onto a Q-Exactive Plus for label-free quantification. The proteome (peptides before acetyl-enrichment) and the acetylproteome data sets were then subjected to a statistical workflow developed for PTM analysis and integrated to identify potential substrates of SIRT1 and diet- and/or tissue-specific acetylation events.
Results
We identified over 1,600 acetylation sites belonging to 719 proteins and their statistical analysis uncovered potential tissue independent and tissue specific SIRT1 substrates. Our integration of the acetylome and proteome data sets shows major SIRT1-dependent changes of the acetylproteome upon nutrient restriction in the liver and muscle.
Conclusions
This work allowed the identification of global acetylation changes due to SIRT1 extinction in function of nutrient availability. We identified previously unreported potential substrates of SIRT1 providing clues on its cellular functions in different organs. Finally, the undergoing integration of our data set with similar studies of other Sirtuins should enlighten on their inter-dependencies in a tissue-specific fashion.

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Validation of novel plasma markers for Venous Thromboembolism using affinity proteomics combined with mass spectrometry

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Background: Venous thromboembolism (VTE) has an incidence of 1-2/1,000 year, high mortality and 25% recurrence rate. Risk prediction and diagnosis remain a challenge, due to the lack of specific markers, as the only plasma marker currently used, D-Dimer, has low specificity.
The aim is to identify and validate novel specific VTE plasma markers and to study their relevance to disease development.
Methods: This work is based on a first discovery screen performed in plasma samples from a Swedish (VEBIOS) study with replication in a French study (FARIVE). VEBIOS sample set contained both patients with acute VTE (n=96) and patients sampled after discontinuation of anticoagulants following a first-time thrombosis (n=192). In FARIVE (n=1200), patients were sampled shortly after diagnosis. 768 antibodies from Human Protein Atlas project and commercial sources targeting 408 proteins were used to profile both cohorts, using multiplex suspension antibody bead arrays. Immunocapture-mass spectrometry (IC-MS) and dual-binder immunoassays were used to validate identified targets associated with VTE.
Results: A number of potential marker candidates significantly associated with VTE were identified. By IC-MS, we verified the binding of these candidates to the antibodies used in the discovery. In particular, we identified a component from the alternative complement pathway. IC-MS also revealed tentative protein-protein interactions between targets and other proteins in plasma. Significant differences (cases vs controls) in the plasma levels of these candidates were established in both VEBIOS and FARIVE ($P<0.001$) by in-house developed quantitative dual-binder sandwich immunoassay, confirming the discovery results.
Conclusions: MS-based techniques, such as IC-MS allowed us to verify the antibody-target candidates from discovery-phase before further validation of markers in a clinical prospective study by dual-binder immunoassays.
Keywords: VTE, biomarker, affinity-proteomics, IC-MS, dual-binder-immunoassays

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Tracking subcellular localisation changes with Dynamic Organellar Maps using SILAC, TMT or LFQ

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BACKGROUND:
We previously developed Dynamic Organellar Maps, a method which combined subcellular fractionation with mass-spectrometry based proteomics and supervised machine learning, to reliably determine protein subcellular localization, and the identification of translocation events in comparative experiments. The use of metabolic labelling for quantification (SILAC) rendered the method best suited to cells grown in culture.

METHODS:
Here we have adapted the workflow to both label-free quantification (LFQ) and chemical labelling/multiplexing strategies (Tandem Mass Tagging, TMT 10-plex). In each case the same subcellular fractionation is performed, either quantifying each fraction using the MaxLFQ algorithm or, labelling peptides with TMT reagent and quantifying with a mass spectrometer capable of SPS-MS3.

RESULTS:
We demonstrate that both new methods are highly effective for generation of organellar maps and capture of protein translocations. Comparison of the three methods reveals that each has its own strengths; accuracy (SILAC), depth (LFQ), or TMT (Speed).

CONCLUSIONS:
This study extends the scope of Dynamic Organellar Maps to any cell type or tissue, and also to high-throughput screening.

KEYWORDS:
Subcell Atlas, Map of the Cell, Spatial Proteomics, SILAC, LFQ, TMT 10-plex, EGF signalling

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Insights into human brain development; A proteomics perspective

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Background:
The development of the human brain starts after conception and continues lifelong, affecting the brains size, shape and most importantly, its intellectual capacity including cognitive, perceptual and motor abilities. Here, we used Mass Spectrometry based proteomics and rigorous statistical analysis to identify proteins expressed in human brain at different post-natal developmental stages. The aim was to gain new insights helping to better understand temporal developmental processes.
Methods:
Post-mortem prefrontal cortex tissue from 69 individuals aged six weeks to 49 years were obtained from individuals that died due to natural causes or accidents and were defined as normal controls by pathologists. Quantitative proteome analysis of trypsin-digested brain samples was carried out using a 1290 Infinity LC coupled to Agilent 6550 iFunnel Q-TOF MS instrument. We identified discrete groups of co-regulated proteins using weighted correlation network analysis (WCNA) and tested them for association to age.
Results:
We detected 952 proteins regulating a wide range of biological processes including, but not limited to, nervous system development, neurogenesis and axon-dendrite development. There were 396 proteins which had less than 15% missing values. From here, WCNA identified three modules with significant positive associations with age and two modules with significant negative associations with age that were not associated with other confounding factors. One module (53 proteins) displaying a gradual increase in protein expression throughout development was enriched for direct protein interactions and implicated in pyridine compound and nicotinamide nucleotide metabolic processes. Another module (61 proteins) displaying a gradual decrease in protein expression throughout development was enriched for direct protein interactions and implicated in axon development, generation of neurons and nervous system development.
Conclusions:
This is the first proteomics study focusing on human brain development at different developmental stages. The protein changes revealed novel insights into the critical stages of cognitive development.

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The winning trio in Metaproteomic: 75 cm column, Orbitrap Fusion™ Lumos™ Tribrid™ and X !TandemPipeline

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Bottom-up approach was fully used in quantitative proteomics strategy. The last development in shotgun approach was mentioned in the article «The one hour yeast proteome» (Hebert et al (2014)), in which 3,977 proteins were identified (1,3 hours of run, 35 cm column, Orbitrap Fusion™Tribrid™). This let us to imagine what we could be able to achieve if we could improve the column size and the time of run with a fast and sensitive mass spectrometer.
PAPPSO platform works since a long time with complex samples in the Metaproteomic field, using 50 cm column with sensitive mass spectrometers (Juste, C. et al. (2014). Metaproteomic samples are extremely complex and have a particular dynamic range, that makes the mass spectrometry analysis more difficult than with others samples. Recently outfitted with an Orbitrap Fusion™ Lumos™ Tribrid™, we have used 75 cm column (Thermo Scientific) to improve the number of identified peptides while keeping an acceptable run time.
Different methods of sample preparations with patient heart disease were tested. This new column allowed to improve by 30 to 50 % the number of identified proteins.
The results will be discussed with X!TandemPipeline (Langella, O. et al. (2017)), a house made software and designed to perform protein inference and to manage the redundancy of peptides identification results after the database search. This software, free and open source is the only one able to deal with very large raw data sets and huge database, yielding possible the treatment of hundreds of complex samples in a short time.
A new generation mass spectrometer, a longer column and efficient analysis software have made available the microbiota analyses of more than 500 patients. This big cohort, allows us to have a better comprehension of the metabolism among ill individuals in order to discover future therapeutics targets against metabolic disorders.

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Development of an Enzyme-assisted Targeted Glycopeptide Quantification Method for Early Detection of Liver Diseases

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Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the third most common cause of cancer death worldwide. Precisely diagnosis of HCC at its early stage provides up to 50% enhancement of survival rate. However, insufficient detection sensitivity of current approaches necessitates the development of a robust analytical strategy with high throughput multiplexed capability for the quantification and verification of protein biomarker candidates. In addition to concentration, altered glycosylation patterns may also provide better sensitivity for disease diagnosis. In this study, we chose alpha-fetoprotein (AFP), especially its core-fucosylated form, as a model analyte to demonstrate the proposed workflow since it has been used as a diagnostic and prognostic marker for HCC. We first profiled intact glycopeptides by a computational tool, MAGIC. Nineteen glycoforms, which are sialylated biantennary with or without core-fucosylation, were confidently identified in human cord blood. To address the issue on the various ionization efficiencies of intact glycopeptides, endoglycosidase, which specifically cleaves the glycosidic bond between two GlcNAc, was used to reduce the microheterogeneity of glycan moiety on peptides. With an aid of the enzyme, the truncated glycopeptides were directly analyzed by SRM and quantified by the peak area of target m/z. The ratio of core-fucosylated to non-core-fucosylated glycopeptide in different patient cohort is easy to obtain after comparison between peak areas. We evaluated the analytical figures of merit of the proposed method, followed by the guideline from US Food and Drugs Administration (US FDA) for bioanalytical method validation. The targeted glycopeptide quantitation had good linearity (r^2=0.9906 for interday experiments) and a limit of detection of 15.6 ng/mL. We anticipate applying the proposed protocol to understand the correlation between glycosylation and liver diseases for early detection purpose.

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Combined Proteomics and Metabolomics Approach for the Investigation of Metabolic Deregulations in Chronic Lymphocytic Leukemia

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Background

Chronic lymphocytic leukemia (CLL) as the most common type of leukemia in adults in the western world primarily affects B lymphocytes and manifests itself by accumulation of non-functional, clonal B-cells in peripheral blood, bone marrow and lymphoid tissue. CLL is still incurable and eventually causes repression of other blood cells leading to symptoms like anemia and thrombocytopenia. While many aspects of CLL have been studied in great detail resulting in novel therapeutic applications, CLL metabolism has been hardly studied and holds great potential for future clinical intervention strategies.

Methods

B lymphocytes isolated from peripheral blood of healthy donors and CLL patients were fractionated into cytoplasmic and nuclear fraction. Proteome profiles were generated via HR-MS and data subjected to MaxQuant software for label-free quantification. Targeted metabolomic analyses of corresponding whole cell lysates were performed using the Biocrates AbsoluteIDQ p180 kit for the quantification of 180 metabolites including acylcarnitines, amino acids, biogenic amines, sphingolipids, monosaccharides and glycerophospholipids.

Results

HR-MS proteomics yielded about 7000 protein identifications for the entire dataset. Relative quantitative comparison between age-matched normal B cells and CLL cells resulted in 426 and 428 significantly regulated proteins (FDR<0.05) for cytoplasmic and nuclear fraction, respectively. Aside from well-known CLL-associated regulations like increased levels of BCL2, many metabolically relevant proteins were found altered in CLL cells. Pathways identified to be particularly affected were glutamine and lipid metabolism. These findings were corroborated on the metabolite level as glutamine and glutamic acid were found to be strongly deregulated within CLL cells along with glycerophospholipids and sphingolipids.

Conclusion

The novel finding of the importance of glutamine and lipid metabolism in CLL may support the development of novel and innovative treatment options for enhanced therapy success in the future.

Keywords

Chronic lymphocytic leukemia, shotgun proteomics, targeted metabolomics, glutamine metabolism, lipid metabolism

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ProteoRE, a Galaxy-based infrastructure for annotating and interpreting proteomics data

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Background: Concurrently with the increased simplicity associated with producing MS-based proteomics data, the bottleneck in many laboratories has shifted to reliable and reproducible interpretation of the data to extract meaningful knowledge. The ProteoRE (Proteomics Research Environment) project is a joint effort between the French proteomics infrastructure (ProFI) and the French bioinformatics Institute (IFB). Its primary aim is to centrally provide the proteomics community with an online research service enabling biologists/clinicians without programming expertise to explore their proteomics data through the Web in a reproducible manner. ProteoRE is built upon the Galaxy framework, a well-maintained software platform, providing simple interfaces to tools and online access to computational resources in a collaborative and transparent way.

Methods: Starting from proteome software output files (e.g. MaxQuant and Proline), various components have been designed driven by expertise and needs from our collaborators (biologists). These components embedded into modules have been implemented either by reusing tools (from the Galaxy Tool Shed) or by wrapping Bioconductor packages and external code, and beta-tested.

Results: We have set up two use cases scenarios derived from our own research projects: the first case consists in interpreting a large proteins identification list while the second entails selection of biomarkers candidates based on biochemical criteria. Current components include customary data manipulation (e.g. filtering, sorting, and ranking), annotation (e.g. information retrieval from public resources), downstream analysis (e.g. cross-comparison and enrichment analysis) and graphical representation. A ProteoRE instance integrating these components and tools, is now deployed for ergonomics assessment before public release in early 2018.

Conclusions: While Galaxy-based tools offers services for primary proteomics data analyses (e.g. MS data conversion, protein database tools, search algorithms), tools focusing on downstream analysis are still lacking. The ProteoRE platform proposes to fill this gap with the hope of promoting proteomics data in the Life Science community.

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Characterization of proteome of vitreous humor in retinal detachment using different experimental setups

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Background: Retinal detachment (RD) is a potentially blinding condition characterized by a physical separation between neurosensory retina and retinal pigment epithelium. DR management is exclusively chirurgical but new therapeutic approaches may improve the visual outcome after surgery. Due to its prime localization, the analysis of vitreous humor (VH) proteome can help to understand the disturbances in retinal function during RD and to found potential biomarkers or pharmacological targets.
Methods: Anion exchange chromatography combined with SDS-PAGE (IEX-SDS-PAGE) and bidimensional reversed phase liquid chromatography (2D-RP-LC) were explored as fractionation strategies to analyze the proteome of a pool of VH from RD patients. For quantitative analysis, iTRAQ labelling combined with 2D-RP-LC and mass spectrometry (2D-RP-LC-MS/MS) was employed to study expression changes in VH proteome of pooled samples obtained from patients with RD (n=4) compared to macular epiretinal membranes (n=4).
Results: A total of 1140 proteins were identified, 1030 through the analysis by 2D-RP-LC-MS/MS and 127 by IEX-SDS-PAGE and MALDI-TOF/TOF. In quantitative analysis, 112 were found differently expressed in RD, 74 overexpressed and 38 underexpressed, compared to control group. By an in-depth analysis of biological mechanisms and functions in which they are involved, overexpressed proteins were found strongly correlated with pathways such as carbon metabolism and biosynthesis of amino acids, while coagulation factors (F9, F11) and complement components (C1R, C8B, C9) were found underexpressed RD. Despite some authors suggest that RRD is an inflammatory occurrence, alpha-1-antichymotrypsin, C-reactive protein, serum amyloid P-component were found downregulated in this study.
Conclusions: By 2D-RP-LC-MS/MS, it was possible to identify a significant higher number of proteins in RD proteome. These results indicate that carbon metabolism, complement and coagulation cascades and biosynthesis of amino acids may be compromised during RD, which provides new insights for understanding of its pathogenesis.
Keywords: Bidimensional fractionation, iTRAQ, Quantitative Proteomics, Retinal detachment, Vitreous Humor.

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The impact of protein thiol redox status on foetal and adult haematopoiesis

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Background
Recent evidence implicates a critical role for redox (reduction/oxidation) regulation and thiol redox status (TRS) in pathways that control normal but also malignant blood cell development. The protein TRS is characterized by the balance in free and oxidized thiol moieties of protein cysteines, which relies mainly on the levels of reactive oxygen species (ROS) and intrinsic cellular regulation. It has been described that mouse foetal liver (FL) haematopoietic stem cells (HSCs) have higher total ROS levels compared to adult HSCs. The aim of this work is to characterize protein TRS in foetal and adult haematopoietic stem and progenitor cells (HSPCs) in order to gain insight into posttranslational modifications regulating foetal and adult mouse haematopoiesis.

Methods
Foetal and adult Lineage- Sca-1+ c-Kit+ (LSK) HSPCs were acquired by FACS sorting of cells extracted from mouse FL in day 14.5 of gestation and adult bone marrow, respectively. After cell lysis, natively reduced protein thiols were labelled with iodoTMT1, the samples were desalted, the reversibly oxidized thiols were reduced using TCEP and newly formed free thiols were labelled with iodoTMT2. Labelled samples were digested and desalted using C18 stage tips. IodoTMT-labelled peptides were enriched using anti-TMT resin and analysed by nanoLC-MS3 using Thermo Scientific Orbitrap Fusion.

Results
For the first time, we successfully applied a sequential iodoTMT labelling method in foetal and adult HSPCs. We found enhanced oxidation of protein thiols in foetal HSPCs compared to the adult HSPCs and described key proteins involved in redox signalling pathways in mouse haematopoiesis.

Conclusions
We propose that redox signalling regulates blood stem cell fate and susceptibility to initiation as well as progression of laeukaemia. Deeper insight into redox signalling will give us a better understanding of childhood and adult laeukaemia.

Keywords
Redox signalling, haematopoiesis, haematopoietic stem cells, proteomics, IodoTMT label

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Integrated investigation into characteristics of proteome, genome and transcriptome of rectal cancer with liver metastasis

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Background Occurrence of liver metastasis is the main cause of colorectal cancer death, and the molecular characteristics have been poorly understood.
Methods In this study, a case of rectal cancer with liver metastasis was recruited, who had two synchronous tumors (the upper tumor and the lower tumor) in the rectum. The tissue samples from the rectal lesions and the liver metastasis were carefully collected, and applied to proteomics analysis, along with genomics and transcriptomics analyses. The tandem mass tags (TMT) quantitative proteomics technique, and next generation sequencing were employed for the investigation.
Results DNA variants suggest that the two rectal tumors were derived from diverse clones, i.e. they were synchronous primary tumors; and that the liver metastasis originated from the upper tumor. RNA profiles demonstrate that the liver lesion was more similar to the upper tumor. Based on the genomics and transcriptomics results, protein differential expression analysis was performed between the upper tumor, or the lower tumor, and the liver metastasis, respectively. Among the significant proteins, there were 8 proteins associated with liver development (ACAT1, AK2, AK4, ALDOB, BAAT, BDH1, CP and QDPR; p = 2.06E-09) in GO, and 14 proteins involved in the PPAR signaling pathway (p = 1.11E-06) in KEGG. Moreover, there were 42 significant proteins that expressed consistently in the upper tumor and liver metastasis, but differentially between the upper tumor and the lower tumor; among them 2 proteins (RPS27A and SORBS1) were enriched in the PPAR signaling pathway (p = 0.0008) in PID KEGG.
Conclusions The integrated analyses of proteomic, genomic and transcriptomic data indicated that the above candidate proteins and their coding genes may have biological and clinical significance for the liver metastasis from colorectal cancer, but further validation will be required.

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ProteomicsDB transforms into an interactive multi-omics platform

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Background
The integration of multiple omics, molecular profiling and phenotypic data sources becomes of increasing importance in both academic and health sectors. The joint effort of TUM, SAP and other partners created a new and publicly available database termed ProteomicsDB, which is based on SAP's HANA in-memory data platform. This unique platform already enables the analysis of proteomic data in real time and provides different analytical features for the interactive exploration but lacks the data model and intelligence for other omics technologies.

Methods
A combined triple-stores (RDF-like) framework brings different omics data to the same point of reference, by storing different resource identifiers in an object-oriented way. This not only enables the automatic mapping of resource identifiers but also facilitates the storage of sematic interactions between those. The new generic data model supports a wide range of quantitative omics technologies. For their combined analysis, different normalization schemes e.g. MCombat, ratio extraction and machine learning approaches were tested to enable the interconversion of different omics expression measurements.

Results
ProteomicsDB is a multi-omics platform that enables the visualization of multiple omics expression measurements across different cell lines and tissues. Expression bars and heatmaps that include hierarchical clustering are dynamically created, while the in-memory platform enables the real-time prediction of any missing values, depending on the existence of at least one omic type for that combination, as well as the real-time statistical analysis and clustering of single or multi omics datasets.

Conclusions
This new release transforms ProteomicsDB into a multi-omics platform enabling the combined analysis of proteomics and transcriptomics data. Users can browse and interact with both data types in the new expression visualization or compare expression levels in a combined heatmap.

Keywords
Bioinformatics, multi-omics, ProteomicsDB, analysis, real-time

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Fate of antigens encoded by self-amplifying RNA vaccines

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Nucleic acid vaccines have been developed to address limitations of both live attenuated and subunit vaccines. Among them, Self Amplifying mRNA vaccine (SAM) has been evaluated in different animal models and has been confirmed to be tolerated and able to drive in vitro antigen expression. However, the mechanism of action of SAM approach has not been fully elucidated. To address this gap, we employed a quantitative mass spectrometry approach to investigate the molecular fate of vaccine antigens encoded by SAM.
In this work, we investigated the quantitative correlation between the antigen expression and epitope presentation on MHC class I molecules in a dose-range and time-lapse assay using myoblast cell cultures, comparing two delivery systems, viral replicon particles (VRPs) and lipid nanoparticle.
The data obtained show that the rate of intracellular antigen expression driven by VRPs is faster compared to LNPs. Moreover we observed a tight correlation between the onset of protein expression and MHC class I epitope presentation for both delivery system, providing strong evidence that epitope presentation is temporally linked to antigen translation. Furthermore, after detection, no evident differences in the intracellular amount of protein antigen and in the level of epitope peptide were observed, assuming that the main difference between VRPs and LNPs is only related to the mechanism of cellular uptake.
Then we applied this technology to quantify the SAM encoded antigen in the muscle of vaccinated mice at the site of injection, where this new type of vaccine are able to generate amount of antigen lower compare to the standard dose given by classical vaccines.
Moreover, we move to an in vitro model of co-colture dendritic cells through, we were able to confirm that this cells are not transfected by SAM but are able to up take antigen encoded by transfected myoblast cells.

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Understanding GSK3β function in cancer cells through precision knockout coupled to high resolution LC-MS/MS.

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GSK3β, a serine-threonine kinase, is a major constituent of the canonical Wnt signalling pathway as part of the Beta-Catenin destruction complex. It is also predicted to have up to a hundred protein interaction partners in its role as a kinase leading to a number of diverse diseases such as cancer, aortic valve calcification, and familial tooth agenesis amongst others. Investigation of functional knockout models of GSK3β is difficult due to its embryonic lethality, and its inhibition in many cell lines results in apoptosis. Here I present the analysis of the cellular proteome in viable isogenic cell lines with a complete functional knockout of GSK3β.

Whole cell protein lysates of a HCT116-derived, genetically disrupted GSK3β cancer cell line and its wild type isogenic partner were quantitatively analysed using LC-MS/MS in MSE acquisition mode on a Synapt G2-S HDMS system (Waters). Raw files were then processed by ProteinLynx Global Server (Waters) and searched against the human UniProt database for peptide identification. Protein quantification was then calculated using the Hi3 method and differentially expressed proteins analysed according to fold change.

Overall 6,282 proteins were identified, of these 1,095 proteins were identified in every sample, with 16 proteins unique to the knockout cell line, and 122 proteins unique to the wild type cell line. Between the two cell lines 268 proteins were found to be differentially, based on a minimum fold change of 2. Analysis of the data illustrates a metabolic shift away from anabolic processes towards catabolic metabolism in the GSK3β knockout cells.

Isogenic cell lines provide a controlled model of protein perturbation in which to accurately assess the remodelling of proteomic signalling networks within an endogenous cellular system. The investigation into the loss of GSK3β in a colorectal cancer environment has revealed global changes in the protein signalling network.

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In-depth quantitative proteomic profiling of rat heart

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Background
The heart is a muscular organ which pumps blood through the blood vessels of the circulatory system. In humans, other mammals, and birds, the heart is divided into four chambers: left and right atria; and left and right ventricles. As the largest and most important component of the human heart, the left ventricle (LV) attracts most research enthusiasm, while other chambers are lack of research, and the proteomic basis of the four-chamber heart is largely unexplored. In this study, we performed the in-depth quantitative proteomics study on rat heart which aimed to find the similarities and differences of protein expressions lying in various chambers and to construct the in-depth rat heart proteome landscape.

Methods
The cardiac four chambers were procured from identical myocardial loci of rat. Heart tissues were grinded under liquid nitrogen and protein were lysed in buffer. After digestion, peptide mixtures were labeled by iTRAQ reagent. The mixed sample was detected by 2D-LC combined Triple TOF 5600 instrument in triplicate.

Results
In this study, we used iTRAQ-coupled 2D-LC MS/MS method to investigate the cardiac proteomic profile and the differences between the chambers in rat heart. In total, 7082 proteins were identified and 4061 proteins were successfully quantified. In addition, we defined the differential protein expression by comparing each chamber with others to further analyze enriched function roles in each chamber (LV vs LA; RA vs LA; RV vs LA; LV vs RA; LV vs RV; RA vs RV). According to the biological processes analysis, ventricles are mostly related to regulation of the contraction of the myocardium, aerobic respiration and energy metabolism, while atrium focus on extracellular structure organization.

Conclusions
We identified 7082 proteins, quantified 4061 proteins and constructed a defined rat heart proteome profile.

Keywords
Rat / heart four-chamber / proteomics/ LC-MS/MS

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An optimised quantitative Multidimensional Protein Identification Technology for in-depth profiling of the human plasma proteome

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Background: Plasma proteomics can portray physiological states and identify dysregulation associated with disease, being a valuable tool for biomarker discovery. However, the wide dynamic concentration range of proteins in human plasma represents a major analytical hurdle. Currently, most proteomic studies are capable to quantify 500-1000 plasma proteins relying upon sample pre-treatment for depleting high abundant proteins. Here, an optimised quantitative multidimensional protein identification method (qMudPIT) based on size exclusion chromatography (SEC) for pre-fractionation was developed to profile human plasma. This approach is currently being applied to define a novel biosignature for diagnosis of active pulmonary tuberculosis.
Methods: Plasma samples were pre-fractionated using SEC and 6M guanidine hydrochloride to produce four segments. Each segment was then desalted, trypsin digested and iTRAQ labelled. A RP-HPLC gradient using C4 chemistry was optimised for pooled labelled-peptides fractionation. Solid phase extraction was used to clean up iTRAQ- labelled peptides. Fractions were subsequently LC-MS/MS analysed. The results were analysed by median-adjusted normalisation of raw peptide intensities, log2 transformation and averaging for protein expression.
Results: Our method was able to identify and quantify proteins with natural abundance spanning 11 orders of magnitude. In total, 4772 proteins were confidently quantified of which 3196 were uniquely profiled in plasma (5% FDR). Of biomedical importance, the offline C4 fractionation facilitated mono and multi-phosphorylated proteotypic peptide identification. As a key methodological step, the chaotropic diluent allowed the effective extraction of proteins encompassed in vesicles innate to non-depleted plasma. As a result, 1583 proteins were annotated as vesicle-enriched from ectosomes and exosomes.
Conclusions: To our knowledge, our qMudPIT approach has generated one of the most comprehensive plasma proteome coverage to date. Such a trait provides an unparalleled opportunity for unveiling new biomarkers, and understand new aspects of the physiopathology of many conditions hidden in the complexity of human plasma.

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Innovative mass spectrometry to assess the clinical value of proinsulin as early biomarker for diabetes

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Background

Diabetes manifests itself in loss of insulin production which may be attributed to pancreatic β-cell stress. Insulin is produced by pancreatic β-cells as preproinsulin, processed intracellularly to proinsulin and subsequently to insulin by proteolytic cleavages. The proinsulin/insulin ratio decreases during development and progression of diabetes. Hence, the proinsulin/insulin ratio may serve as a good biomarker to reflect pancreatic β-cell damage that forms the basis of diabetes. Current diagnostic methods to determine proinsulin levels are affinity-based immunoassays which may yield ambiguous results due to insulin proteoform cross-reactivity.

Methods

We are developing an efficient analysis workflow that quantifies all relevant insulin proteoforms in one scan: proinsulin, insulin and the C-peptide, using specific immunopurification and top-down proteomics analysis. Synthetic proinsulin and insulin protein standards and in vitro stimulated proinsulin production models were used to assess specificity and sensitivity of this approach. LC-MS/MS measurements were performed on a maXis 5G Qq-TOF tandem mass spectrometer coupled to an UHPLC nanoflow liquid chromatograph (Bruker Daltonics). Acquired raw data were processed in DataAnalysis 4.2 and exported to mzXML. Monoisotopic peak picking and charge deconvolution were performed in MsDeconvGui and saved as MSAlign+ input files. Subsequently, TopPIC software was used to match acquired MS/MS data against the UniProt Homo sapiens database.

Results

Antibody binding characteristics were validated on proinsulin and insulin protein standards by Western Blot and intact tandem mass spectrometry. Spike-in recovery assays were performed in a plate-based assay format to validate the efficient immunopurification. A prototype assay was developed whose sensitivity and specificity was tested on in vitro-stimulated proinsulin production models.

Conclusions

This approach overcomes the limitation of affinity-based immunoassays via exact characterization of insulin proteoforms, which enables us to validate proinsulin or the proinsulin/insulin ratio as biomarker for the onset of diabetes in well-characterized patient cohorts.

Keywords: Top-down proteomics, Immunopurification, Diabetes, Biomarker, Insulin

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Molecular Imprinted Polymers (MIPs) for the detection of low abundance proteins as biomarkers for NSCLC

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Background
Lung cancer is the most common cause of cancer related death. Poor prognosis is principally due to patients presenting with metastatic disease. Survival could be improved by early diagnosis. Many potential biomarkers are present at sub ng/mL levels in biological samples. These levels render them undetectable by current methods as they are obscured by highly abundant proteins due to the large dynamic range of protein. KRAS mutations commonly occur in early stage non-small cell lung cancers (NSCLC). We aim to develop a method to detect low abundance proteins such as KRAS and identify mutations as biomarkers for NSCLC. MIPs will be used to enrich the protein in biological samples, enabling its detection by mass spectrometry. This could be used as a quick, non-invasive test to detect early stage NSCLC and inform treatment.

Methods
MIPs were produced by polymerising an acrylamide monomer around a KRAS C-terminal peptide. After the peptide template was removed, the MIPs were incubated in patient plasma with known KRAS status. Other proteins were removed using PBS whilst KRAS remains bound to the MIPs. KRAS was eluted from the MIPs then tryptically digested. Samples were injected onto a NanoAcquity UPLC connected to a Waters Xevo TQ mass spectrometer. KRAS mutations were distinguished from wild-type using a selected reaction monitoring (SRM) method.

Results
MIPs were immobilised on glass beads and separated from plasma proteins using Spin X filters due to KRAS sticking to MWCO filters. A test peptide was successfully bound and eluted and the method was optimised to improve sensitivity. Initial results in plasma will be presented.

Conclusions
MIPs enable the detection of mutant KRAS in plasma. It is hoped that MIPs can be developed for a panel of biomarkers to screen for NSCLC leading to early diagnosis and improved survival.

Keywords
MIPs, lung cancer, mass spectrometry

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Flexible data analysis for label-free time course proteomics experiments

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Background
Many biological processes are dynamic, so time-course omics experiments are essential for further unraveling them. Despite their merits time-course data impose many computational challenges and regularization is typically required to retrieve a smooth representation of noisy observed temporal protein expression profiles. Splines are popular to model non-linear trends in omics, but they have not been adapted towards peptide-based models for label-free differential proteomics, yet.

Method
lmms for instance incorporates splines but it starts from summarized protein expression values, which suffer from bias and/or unequal variance in a label-free proteomics since different peptides and a different number of peptides are typically identified in each MS-run.
Peptide-based methods, e.g. MSstats and MSqRob, do account for peptide-specific effects and immediately provide model-based summarization at the level of the fold change estimates, but their current implementation does not allow for nonlinear trend estimation.
They use factorial models where every time-point is treated as a separate level. Hence, they cannot exploit information in neighboring time-points when estimating trends and are limited to studies that are evaluated on a fixed grid of time-points.
In this contribution we extend MSqRob, our peptide-based method for label-free differential proteomics by exploiting the link between splines and mixed models.

Results
We illustrate our method in case-studies assessing effects of stimuli over time. Our method can detect non-linear trends in protein expression within a single biological condition as well as differences in temporal expression profiles between conditions.

Conclusion
Our extension improves upon lmms and MSqRob by estimating non-linear trends from peptide-level data. It accounts for peptide-specific effects, multiple subject-specific random effects as well as for other confounders.
Hence, our approach can handle longitudinal studies with complex designs where each individual is observed on multiple (unequal) time-points while avoiding peptide-summarisation bias.

Keywords
Splines, nonlinear trends, peptide-based quantitative proteomics, longitudinal proteomics

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N-GLYCOME CHANGES IN CHO CELLS DUE TO EXTRACTABLE AND LEACHABLE EXPOSURE FROM SINGLE-USE BIOREACTORS

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Background: The implementation of single-use bioreactors as alternatives to stainless steel used in the manufacturing of recombinant therapeutic proteins introduces new materials into the bioprocessing pipeline. These plastics can degrade and leach breakdown products into the culture media such as bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP).
Due to the importance of product quality and cell performances in the biomanufacturing process, knowledge of the effects of these leachables compounds is essential. In the present work the consequences of bDtBPP exposure on CHO cell growth have been evaluated using a mAb1 producing cell line (CHO DP12).
Methods: Cells have been cultured in media spiked with bDtBPP and collected during exponential and stationary phases of growth to obtain protein mixture. N-glycans were released from proteins, fluorescently labelled and analysed by 2D weak anion exchange x hydrophilic interaction liquid chromatography hyphenated with mass spectrometry. A label free data comparison with a control was used to highlight the differences between the two samples in both phases of growth. Critical quality attributes for produced mAb1 were evaluated via LC or LC-MS.
Results: Results showed the effect of bDtBPP at concentrations commonly leached from single-use bioreactors, with a decrease ~50% of viable cell density, while viability seemed not to be affected. Moreover, evaluation of the impact of bDtBPP exposure on the quality of produced mAb1 has been performed, showing no differences from mAb1 produced by control cells. Modifications in the N-glycans profile have been analysed, as they are crucial in most cell mechanisms; results showed differences that are more emphasized in the exponential phase of growth.
Conclusion: bDtBPP has a detrimental effect on CHO cell lines probably caused by mechanisms involved in the initial phase of growth. Nevertheless, product quality doesn’t seem to be affected by this leachable compound.
Keywords: CHO cells, N-glycans, LC-MS, extractables, IgG

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The novel role of ADAM17 cytoplasmic domain in modulating redox state via Thioredoxin-1

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Background

Previously we have demonstrated that Trx-1 interacts with ADAM17 and that ADAM17 is negatively regulated by binding to Trx-1 (Aragão et al., 2012). The aim of this work was to investigate the mechanism of regulation of ADAM17 on Trx-1.

Methods

To test the hypothesis of the regulation mechanism of ADAM17 on Trx-1, there were performed the following methodologies: Limited proteolysis (LiP) assay, solid phase binding assay, H2O2 levels measurements, analytical ultracentrifugation, reduction of Di-E–GSSG by Trx-1, measurements of reactivity of cysteines, ADAM17 activity by AP reporter assay, western blot analysis of Trx-1 monomeric state and iodoTMT and TMT labeling. The experiments were performed with recombinant proteins and cells transfected with the cytoplasmic domain of ADAM17 wild type (ADAM17cytoWT) or the mutant form (ADAM17cytoF730A).

Results

The results revealed that the mutant K72A promotes a conformational change and the disulfide formation at the residue C73 of the thioredoxin-1 molecule favors the dimeric and inactive form of the enzyme. Interaction between ADAM17cytoWT and Trx-1 is important for Trx-1 activity and for the monomeric state of Trx-1. It was also concluded that the presence of ADAM17cytoWT leads to more HB-EGF shedding and H2O2 levels. The reduction of Di-E–GSSG by Trx-1 in the presence of ADAM17cytoWT is greater than in the presence of ADAM17cytoF730A showing a more activated Trx-1 when in the presence of ADAM17 wild type. The monomeric state is induced by ADAM17cytoWT and is less abundant in ADAM17 knockdown cells. Finally, the results demonstrated that the overexpression of ADAM17cytoWT promotes a greater abundance of proteins containing free cysteine residues compared with ADAM17cytoF730A.

Conclusions

These results demonstrate that ADAM17cyto can drive the redox switch of Trx-1 and other intracelullar proteins, which open mechanistic insights into the cross-talk between ADAM17 and Trx-1 in redox homeostasis.

Keywords

ADAM17, Thioredoxin-1, mass spectrometry, redox signaling.

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Glycopeptide analysis to examine the role of chlamydial protease-like activity factor

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Background

Recently proteomics studies have been performed to examine the role of chlamydial protease-like activity factor (CPAF), a secreted virulence factor and its role in immune evasion. However, experiments thus far have ignored glycopeptides that are prevalent in the sample as large scale intact glycopeptide analysis remains challenging by mass spectrometry. Here, we perform large scale intact glycopeptide analysis to examine the role of CPAF targets and derive additional insights from glycoproteomics experiments.

Method

Hela 229 cells were infected with Chlamydia trachomatis L2 RST5 CPAF-sufficient strain and RST17 CPAF-deficient strain. Proteins were extracted, trypsin digested and enriched for glycopeptides. Samples were analyzed on Orbitrap Fusion Lumos MS. Data analysis was performed using Proteome Discoverer with Byonic node.

Preliminary Data

In proteomics experiment ~40-50% of MS/MS spectra are identified. Thus, researchers have focused on developing software algorithms to sequence remainder of the spectra with the hypothesis that these are indeed identifiable spectra from conventional peptides. This assumption ignores the fact that the spectra are the result of PTMs. We have observed some of these unidentified spectra are from glycopeptides. Unfortunately, conventional fragmentations are not ideal for glycopeptide sequencing. Further exasperating the issue is that these glycopeptides are present in low abundance. In our proteomics experiments we observed that 10% of our MS/MS spectra were from glycopeptides. XICs of m/z 204.087 (HexNaAc oxonium ion) were performed on LC-MS/MS runs. As precursor ions containing the m/z 204.087 could not be sequenced, these were targeted in a glycoproteomics experiment. Overall we identified over 3000 unique intact glycopeptides in a single LC-MS/MS run and over 5000 in triplicate runs, translating into over 600 unique glycoproteins.

Conclusion

To our knowledge this is by far the largest number of intact glycopeptides reported in a single experiment.

Key words

Glycopeptide, Chlamydia trachomatis, glycoproteomics

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Better access to intact proteoforms by cold vaporization of tissues with picosecond infrared laser ablation

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Background
Physiological processes are regulated by PTMs and disturbances in PTM-processing can cause diseases. During tissue homogenization, endogenous enzymes released can change the original proteform composition. Picosecond-infrared-laser (PIRL) irradiation transforms tissues into aerosols via cold vaporization [1]. Due to the ultrafast transfer of proteins from tissues via gas phase into frozen condensates, we hypothesized that intact proteinforms are exposed to enzymatic degradation reactions to a lesser extent compared to mechanical tissue homogenization. To confirm this hypothesis, protein extracts from tissues obtained by conventional tissue homogenization and by PIRL ablation were investigated by differential proteomics.

Methods
Tissues were homogenized either mechanically (MH-mechanical homogenization) or by PIRL ablation (PH-PIRL homogenization). Mechanical homogenates and PIRL homogenates were separated by 2DE and SDS-PAGE, followed by quantitative LC-MS analysis. Relative degree of proteolysis was determined by proteoform SDS-PAGE migration profiles by plotting peptide intensities against the molecular weight.

Results
2-DE gel analysis indicated that PIRL yielded a higher number of intact proteoforms compared to mechanical homogenization. These results were confirmed by SDS-PAGE migration profiles. Relative degrees of proteolysis were significantly lower in case of PH (2%) compared to MH (23%). These results were further confirmed by spiking experiments. Total yield of intact alpha-casein proteoforms and number of identified phosphopeptides were significantly higher using PIRL, whereas MH showed significantly higher amounts of enzymatically degraded proteoforms. Furthermore, PIRL provides a higher reproducibility and significantly higher protein identification rates.

Conclusions
Due to the short exposure time to enzymatic reactions and the almost particle-free homogenates PIRL leads to significantly higher yields of intact proteoforms and reproducibly identified proteins compared to conventional tissue homogenization. Thus, PIRL therefore provides a previously unavailable access to tissue proteomes at the proteoform level.

Keywords:
PIRL; proteoforms; tissue proteomics; sample preparation

[1] Kwiatkowski et al. Angew Chem Int Ed Engl. 2015 Jan; 54(1):285-8.

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Integrated data analysis pipeline for meta-omics

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Background
While metagenomic sequencing revealed important properties of microbial communities, other meta-omic provides additional insights of the microbial communities. Due to the complexity of metagenomic samples, the main challenges remain in analyzing metaproteomic data. In this study, we designed and compared different strategies of analyzing metaproteomic data when matched meta-omics data are available.

Methods
Metagenomics and/or metatranscriptomics sequencing are assembled and three subsets are obtained: contigs, unassembled reads and assembly graph. FragGeneScan is used to predict proteins from the former two subsets. Graph2Pep/Graph2Pro is utilized to exploit the graph structures of the assembly graphs to predict peptides/proteins for two rounds database searching. We use SOAPdenovo2 for the assembly and MSGF+ for proteomic peptide/protein identification.

Results
We tested the pipeline by two experimental datasets from recent multi-omic studies. In particular, Dataset I was obtained from wastewater treatment tank. Dataset II contained the data from two ocean locations. The preliminary result showed that pipeline improved the metaproteomic identification significantly. Take one sample, BSt 45, as example. In total, there are 90,062 spectra. If only use FragGeneScan to compile a target database from assembly for identification, only 1,892 PSMs (2.1% of total spectra) could be identified at given 1% false discovery rate with 817 unique peptides. Based on the database predicted from the graph structures by Graph2Pep, 6,077 PSMs (6.75%) are identified with 2,412 unique peptides. After Graph2Pro predicted the protein sequences for identification, the number of identified PSMs are increased to 12,748 (14.13%) and 4,542 unique peptides.

Conclusion
Application of our pipeline to two publicly available meta-omics datasets showed that utilizing assembly graph of sequencing reads can significantly improve the characterization of proteins/peptides. We also demonstrated that integration of meta-omics datasets can be used to identify and verify genomic variants in the microbiome.

Keywords
Metagenomics, Metatranscriptomics, Assembly, de Bruijn graphs, Genomics variants

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Links between cell wall remodelling and metabolism in E. coli, a proteomic view.

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Cell wall (CW) hydrolysis is essential for bacterial growth. A set of three redundantly essential D,D-endopeptidases (MepM, MepS, MepH) was recently identified in the Gram negative Escherichia coli. The three enzymes differ with regard to their localization and their abundance. Futhermore, their levels vary in response to the same signal. Therefore, we assumed that their roles may differ in the cell.
To get more insights into the role of CW hydrolysis during bacterial growth, we performed extensive phenotypic, large scale proteomics and CoIP-MS analysis.
Large scale proteomics of WT versus mutant strains deleted in different CW hydrolases in diverse growth conditions differing in carbon source and under oxidative stress was performed. Differential analysis was performed using a new dedicated R pipeline, and interpretation of results was achieved by combining functional analysis tools. Additionally, CoIP-MS was performed to highlight partners of CW related hydrolases in different subcellular locations of the cell. For this purpose, the MaxQuant-Perseus tools were used.
Our phenotypic analysis indicated that deletion of certain hydrolases results in growth defects with particular carbon sources. By combining several proteomics strategies, we revealed partners of CW related enzymes in different compartments of the cell. We identified that deletion of certain hydrolases imposes a re-routing of cellular metabolism. We are currently evaluating how this comes about. We are also looking to confirm protein partners of CW hydrolases and further assays will be exploited to validate the interaction and understand the molecular details of the involved mechanism.
We are currently studying the role of important CW related enzymes in gram negative bacteria during growth. Extensive proteomics analysis highlighted new key roles in physiological metabolic pathways for CW hydrolytic enzymes. We consequently identified for the first time possible moonlighting functions for CW related enzymes.

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Comprehensive glycopeptide profiling in blood plasma for clinical applications

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Background

More than 90% of all proteins in blood plasma carries glycan structures that impact protein function. Glycoproteins are regarded as high potential biomarkers in blood plasma since aberrant glycosylation is known to occur in various diseases. At present, the glycosylation status of proteins in blood plasma can be determined via released N-glycan profiling or targeted analysis of a single glycoprotein. However, these are incomprehensive analyses, not covering the unique combination of glycosylations and protein moiety of multiple proteins. Glycopeptide profiling offers the potential to generate site-specific glycosylation profiles for hundreds of proteins in a single experiment. To this end, we have developed an innovative glycopeptide profiling approach for blood plasma.

Methods

Blood plasma was obtained from healthy individuals and selected patients with congenital disorders of glycosylation (CDG). Samples were subjected to tryptic digestion and glycopeptides were enriched using Sepharose material for analysis by C18RP LC-MS/MS (maXis 5G and Impact II, Bruker Daltonics). In-source charge manipulation was performed using organic solvents (nanoBooster, Bruker Daltonics). Data dependent CID MS/MS spectra were recorded with optimized settings that favor glycan- or peptide-moiety fragmentation. Quantitative LC-MS data was processed in OpenMS. Glycan moieties were identified in GlycoQuest (Bruker Daltonics) and peptide moieties in MASCOT (MatrixScience). Matlab (MathWorks) scripts were developed to integrate all data and to identify differential glycopeptides by multivariate data analysis.

Results

More than 10.000 unique deconvoluted monoisotopic features were detected at >75% group count between samples. Results obtained by glycopeptide profiling were validated by comparison with released N-glycan profiling and intact glyco-Transferrin LC-MS. Multivariate analysis unambiguously classified healthy individuals from patients with known CDG gene defects. The variable importance in projection was used to identify glycopeptide biomarkers for specific gene defects.

Conclusions

This approach enables comprehensive glycopeptide profiling in blood plasma for clinical applications.

Keywords

Glycoproteomics, Glycopeptide, Biomarker, Blood plasma, Supercharging

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Application of novel SILAC analysis software Proteolabels for large-scale meta-analysis of phospho-proteomes

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Background
Stable isotope labelling can be routinely applied in LC-MS proteomics, for large scale accurate and reproducible quantitative profiling. Labels are incorporated metabolically, enzymatically, chemically or by stable isotope. We have developed new quantitative analysis software called Proteolabels extending the functionality of Progenesis, as well as analysing label-free natively, it can process stable isotope labelled data. Here we demonstrate utility of the software for re-analysing public domain phospho-proteomics data on a large-scale using a set of consistent, highly controlled quality metrics. Results provide a standardised dataset for understanding coordination in phosphorylation signalling.

Methods
Human datasets reporting on stable isotope labelled proteomes were downloaded from ProteomeXchange. Data were processed through Progenesis for feature detection, LC-MS map-alignment, deconvolution, centroiding and peak list generation. Identification was performed via Mascot using identical parameter sets for each instrument, across different samples. Proteolabels was used to quantify peptide pairs/triples following default parameters, and ensuring <1% peptide-level FDR identification throughout. Fold change values and confidence intervals were exported, along with protein descriptors for each peptide for downstream analyses.

Results
Fold change, normalised to a consistent scale, were used to construct co-expression networks for phospho-peptides. Co-expression networks show those biological entities (i.e. phospho-peptides) demonstrating correlated changes in expression across different conditions. The networks constructed will enable exploring the extent to which phospho-peptides believed to be acting in the same signalling processes are co-regulated. Phospho-peptides mapped to the same protein chain are also of interest to discover those isoforms appearing to be under coordinated control (and thus highly correlated), as compared with other sets of peptides within the same protein or group of isoforms showing no correlation - indicative of involvement in different biological processes.

Conclusion
Meta-analysis of publicly available phospho-proteomics data enabled the construction of co-expression networks to study coordination in signalling events.

Keywords
SILAC, Dimethyl, Label-based

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Proteome-wide identification of ADP-ribose acceptor sites in mammalian cells and mouse tissues

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Background
ADP-ribosylation is a reversible post-translational modification (PTM) that is catalyzed by ADP-ribosyltransferases (intracellular ARTDs and ectopic ARTCs) and results from the transfer of an ADP-ribose (ADPr) moiety from NAD+ to specific amino acid residues on target proteins (MARylation) or to ADPr itself (PARylation). Targets are modified during a variety of cellular stress conditions such as oxidative or genotoxic stress.

Methods
We established a mass spectrometry (MS) workflow that includes reduction of PARylation to MARylation and affinity enrichment of MARylated peptides. Moreover, we developed an advanced MS methodology for the accurate localization of ADPr acceptor sites, which is based on ADP-ribose marker ion product preview scans and subsequent HCD and EThcD fragmentations.

Results
We identified several hundred ADPr acceptor sites in oxidative stress-induced HeLa cells. These proteins are mainly involved in chromosome organization and RNA/DNA metabolism. To dissect the contribution of the nuclear ARTD1 and ARTD2, we measured H_2O_2-induced ADP-ribosylome(s) in cells with stable ARTD1/2 knockdown. We identified ARTD1 to be the predominant ADP-ribosylating enzyme with specificity for serine residues.
Because of its potential clinical relevance, we mapped the ADP-riboslyome of heart tissue under basal and inflammatory conditions. We detected high levels of arginine modification already under basal conditions, particularly on proteins with a high enrichment in extracellular and plasma membrane protein fractions. We identified the GPI-anchored ADP-ribosyltransferase 1 (ARTC1) as the responsible enzyme.

Conclusions
We established an ADPr-specific MS-based workflow for the detection of ADP-ribosylated proteins in cells and tissues. Under oxidative stress, ARTD1 was the main nuclear enzyme ADP-ribosylating serine sites. ARTC1 has emerged as a major ADP-riboslyation modifier in the mouse heart. Several of the identified modified targets play a role in inflammation and their modification might interfere with extra- to intracellular signaling.

Keywords
PTM, Protein ADP-ribosylation, PARP, EThcD

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Profiling Biochemical Individuality: Human Personal Omics Profiling (hPOP)

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Recent advances in high throughput technologies allow profiling of thousands of analytes within a single experiment. These measurements could potentially be used to diagnose disease early, monitor treatment progression and stratify patient groups to ensure each individual obtains the treatment best suited to their needs. This personalized approach to medicine would include continuous monitoring of thousands of parameters over a whole lifetime. However, in order to be able to interpret such data, we need to have a better understanding of the underlying natural variation of biological molecules in large crowds. If we know the natural ranges of individual analytes, the expected responses to perturbations and the long-term trends in their levels, we can draw meaningful conclusions from comprehensive personalized profiling.
We were able to successfully run a pilot study on human Personal Omics Profiling (hPOP) during 2016 US HUPO conference in Boston and then launched the study at 2016 HUPO. Overall about 150 individuals participated in the study so far and their urine, stool and blood samples have been collected. Our preliminary data on plasma proteins quantified suggests large amount of variance in the data and there is variance we currently cannot explain. With little technical variance, individual variance is large in certain proteins.Using our current SWATH-MS, in total 530 proteins with 1%FDR in peptide level were quantified over 31 subjects. Over 80% of all proteins showed less than 10% CV in SWATH data on hPOP pilot plasma samples whereas when we add technical and biological variance, the 80% of all proteins have over 50% variance. Overall there is 46.5% biological variance which includes the unknown genetic components and unknown environmental components and then there is the 21% of the variance (on average) that we can explain with variables such as age, sex, BMI and ethnicity.

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Understanding the protein corona of nanocarriers is a prerequisite for targeted drug delivery

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Background

For successful biomedical application of nanocarriers, the targeted delivery of a cargo (drug) in vivo is essentially. However, as soon as nanomaterials enter the blood stream, proteins immediately cover its surface, forming the so called protein corona. Thus, highly affects the biodistribution, cellular uptake and toxicity of nanomaterials. In order to prolong blood circulation and prevent unspecific uptake by phagocytes, it is today´s gold standard to modify the surface of nanocarriers with polyethylene glycol (PEG) herby providing ´stealth´ properties. On top of this, the surface is functionalized with targeting moieties (antibodies). Using label-free mass spectrometry we deeply analyzed the protein corona in order to understand the nanocarriers´ behavior in vivo and improve their properties for therapeutic applications.

Methods

Polymeric nanoparticles were covalently and non-covalently modified using PEG in order to obtain stealth properties. After exposure to human plasma, protein corona coated nanocarriers were isolated and the protein composition was quantitatively analyzed by mass spectrometry. Cellular interactions with phagocytic cells (macrophages, dendritic cells) were analyzed by flow cytometry.

Results

Proteomic analysis indicated that distinct proteins adsorb on the surface of PEGylated nanocarriers. It was found that especially apolipoproteins - Clusterin and ApoAI - are highly enriched in the protein corona of stealth nanocarriers. It was shown that those proteins have cell-repellent properties as cellular uptake towards macrophages (RAW264.7) was strongly decreased. This indicated that distinct plasma proteins are necessary to mediate the stealth effect. By artificially decorating the nanocarriers´ surface with those proteins, we showed that we can tune the nanocarriers´ properties.

Conclusions

As protein corona formation is inevitable it is essentially to analyze the composition. This knowledge is necessary to improve the properties of nanocarrier and opens up new strategies to exploit the protein corona in order to design successful targeted delivery systems.

Keywords
Nanomedicine, protein corona, stealth, targeting

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Old dog with new tricks: Tear proteome changes associated with renouncement of contact lenses

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Contact lenses (CL) are an attractive alternative to the use of spectacles. Although beneficial, there are several adverse effects on the ocular surface attributed to CL use, especially perturbations in tear film dynamics. To date, alterations associated with renouncement of CL use are unknown. Therefore, this study elucidated for the first time the tear proteome of CL users following renouncement of CL.

Tear samples were collected from non-CL users (control, n= 22), hard (n= 16) and soft (n= 18) CL users before and after renouncement (for 4.7 ± 0.7 days) of CL wear. Label-free quantitative proteomics based on one-dimensional gel electrophoresis combined with the LC-ESI-LTQ-Orbitrap MS system was employed to assess the differential expressions of tear proteins. The acquired MS spectra were analysed by MaxQuant computational proteomics platform, followed by statistical analysis employing Perseus software and, functional annotation and pathway analyses.

In total, 261 tear proteins were identified with less than 1 % false discovery rate, and 93 proteins were significantly (P <0.01) differentially expressed in both CL groups. A significant number of differentially expressed proteins was related to inflammatory, metabolic and apoptotic processes. Differential expressions of certain clusters of proteins were dependent on the lens type, namely the downregulation of CST1 and PRR4 and, upregulation of TF and CSTA in the hard CL. In the soft CL, PROL1 was down- and SERPINA1 was up-regulated. Remarkably, many of these, namely MSLN, TF and PIP reverted to near-normal levels following CL renouncement. Conversely, specific protein clusters were not restored back to normal levels after renouncement, such as ANXA1, ANXA2 and FABP5.

Conclusively, this study unravelled several novel aspects in the tear proteome changes associated with CL renouncement. Specific clusters of proteins identified in each CL group represent potential clinical biomarkers indicative of breach of tear homeostasis.

Tears, contact lenses, discovery proteomics

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Proteomic analysis of T cell activation-dependent changes in NF90 and bound histones

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Background
T cells coordinate the immune response. Effective T cell activation is central to normal host defense against invading pathogens, as well as surveillance and eradication of intrinsic malignancies such as cancer. Nuclear Factor 90 (NF90) is a DNA- and RNA-binding protein that regulates inducible gene expression of Interleukin 2 (IL-2) upon T cell activation, on the levels of transcriptional activation, post-translational mRNA stabilization and nuclear export. Phosphorylation on serine, threonine, and tyrosine residues is an important modulator of protein function and signal transducer of extracellular stimuli. How NF90 is regulated to induce IL-2 expression upon T cell activation is currently unknown.
Methods
Jurkat T cells were stimulated and extracts were enriched for nuclear proteins. Immunoprecipitation was used to purify native NF90 protein from activated Jurkat nucleus. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed to reveal binding partners in the nucleus, and T cell activation-dependent post-translational modifications on NF90.
Results
NF90 is substantially phosphorylated at multiple sites in vivo. T-cell stimulation induces new phosphorylation of NF90 on tyrosine 365 and threonine 596, in proximity to a putative nuclear localization sequence. Remarkably, NF90 is associated with core histones in resting and stimulated T-cells, and T-cell stimulation augments this interaction of NF90. Acetylation on histone 3 lysine 27 (H3K27ac), an epigenetic mark associated with active enhancers, was detected in association with NF90 only in activated T-cells.
Conclusions
NF90 is a DNA- and RNA-binding protein that regulates gene expression on multiple levels. Immunoprecipitation followed by LC-MS/MS revealed novel physical association with core histone proteins in the nucleus. Proteomic analyses suggest how T-cell activation signaling may be transduced through NF90 to regulate inducible expression of genes including IL-2.
Keywords
NF90, histones, T cell activation, IL-2, phosphoproteomics

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Quantitative Proteomic Analysis of Drug Transporters in Human Blood Brain Barrier

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Background: The quantification tissue expression of drug transporters is important in pharmacokinetic studies to understand the disposition of drugs and their metabolites and predict drug clearance and drug-drug interactions. Changes in transporter expression are thought to be implicated in various conditions, including Alzheimer's disease (AD). Recent advances in quantitative proteomics can enable measurement of transporters in the blood-brain barrier (BBB), and therefore can be used to develop pharmacokinetic models for psychoactive drugs. This study aimed to implement global and targeted proteomic approaches to quantify the main drug transporters in human brain microvessels.

Methods: Brain microvessels were isolated from brain tissue (2.5-3.0 grams) from 12 healthy, 8 Alzheimer's and 2 dementia patients. A QconCAT with selected peptides from target transporters was used as an internal standard for quantification using LC-MS/MS. Spectroscopic methods were used to determine the individual protein content of microvessel fractions per gram brain.

Results: Brain microvessel protein content was determined in healthy and disease samples as 0.27 and 0.36 mg/gram brain, respectively. The purity of isolated microvessels was demonstrated using brain endothelial markers. Targeted and label-free quantification demonstrated similarities and differences in transporter expression levels between healthy and AD brains. For example, a similar level of ABCB1/P-gp/MDR1 was shown in AD (6.26 pmol/mg) and healthy (6.54 pmol/mg) donors. By contrast, ABCG2/BCRP was below the limit of quantification in AD samples compared to expression levels at 8.27pmol/mg in healthy samples. Similarly, ABCC2/MRP2 was not detected in AD samples and measured at low levels in healthy donors (0.35 pmol/mg).

Conclusions: Preliminary data demonstrated differences in expression levels of transporters in the BBB of healthy and AD patients. These data can be used as scaling factors for brain in vitro in vivo extrapolation (IVIVE) and pharmacokinetic modelling in AD research.

Keywords: Drug transporters, Protein quantification, QconCAT, Alzheimer's disease (AD)

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Ultrasensitive Glycoproteomics Pairing LP-IEF with UPLC-MSE to Identify Site-Specific N-Glycosylation Changes in Gastric Adenocarcinoma

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Background: Gastric cancer (GC) is the third most common cause of cancer death worldwide with poor early stage detection necessitating the need for early stage GC specific biomarkers. Global glycoproteomic approaches provide a wealth of information when identifying potential diagnostic biomarkers. This study applies an ultrasensitive serum glycoproteomics platform that combines quantitative glycomics and in-depth quantitative proteomics studies to reveal site specific N-glycosylation alterations in gastric adenocarcinoma.

Methods: Serum samples from patients with GC before (Pre-Op) and after (Post-Op) tumour resection were depleted of their 14 most high abundant proteins and fractionated using liquid phase isoelectric focusing (LP-IEF). Released N-glycans were analysed using UPLC-HILIC-FLR while peptides and glycopeptides were analysed using data independent nanoUPLC-MSE. A bioinformatics pipeline in 'R' for in-silico identification of potential glycopeptides was developed for differential site-specific N-glycopeptide analysis by incorporating glycomic, proteomic, and glycoproteomic data outputs.

Results: Over 100 glycans including sialic acid isomers were identified revealing differential expression between the Pre- and Post-Op states. Proteins were identified to sub-femtomolar concentrations with over 80 proteins found to be differentially expressed between the Pre- and Post-Op states. Identified proteins were found to be involved in a multitude of biological functions with some originating from cellular components. Site-specific glycopeptide identification revealed the presence of differential glycan expression on glycopeptides including glycopeptides from proteins not found to be differentially expressed.

Conclusions: In-depth mining of the serum matrix enables identification of low abundant proteins including cellular proteins secreted into blood. UPLC-HILIC analysis of N-glycans released from fractionated biological samples provides comparative glycomic analysis at a level more sensitive than untargeted global glycomic profiling. Site-specific N-glycan occupancy within identified glycopeptides and glycoproteins reveals the presence of differential glycan expression on glycopeptides. This process provides a great level of information for better identification of potential diagnostic biomarkers.

Keywords: Proteomics, Glycomics, Glycoproteomics, UPLC-MSE

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Toward a true quantitative protein atlas

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Due to the technical limitation, currently a true absolute quantitative proteomic database at population level is still a missing node in human proteomics project. We attempted here to build a true absolute quantitative proteome reference in a small population with the recently developed high throughput technique, Quantitative Dot Blot method (QDB) (Oncotarget, 2017).at a reasonable price and effort. As a pilot experiment, we were able to measured CAPG level in prostate tissues of both wild type and TRAMP mice using a recombinant CAPG protein as protein standard. We further extended our study to human kidney cancer to measure the absolute levels of several novel potential kidney cancer biomarkers (including LMP7, ANXA4, PYGL etc) as well as tubulin using recombinant proteins as protein standards respectively.

Using a recombinant CAPG protein, we were able to detect as little as 3 pg of CAPG protein in QDB analysis, with R2 at 0.994. The reliability and sensitivity of QDB analysis were further confirmed by repeating the same experiment by different operators at different times. The absolute levels of LMP7, PYGL, ANXA4 and tubulin in human kidney cancers were also measured in high throughput format. Our results demonstrated the first proof-of-concept study towards a quantitative protein atlas at population level at low cost and high efficiency. We propose the true quantitative protein atlas as a growing database to compare, communicate and combine with databases built on the same concept across the regions and areas worldwide. This concept of true quantitative protein atlas may unit all efforts in proteomic research to make a significant impact on biomedical and biological research in near future.

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Proteomic Identification of Vitreous Biomarkers for Retinal Degeneration

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Background
Retinal biopsy procedures are invasive and carry high rates of visual morbidity. Therefore, it is not feasible to routinely biopsy the neurosensory retina and monitor molecular biomarkers associated with retinal degenerative. To overcome this hurdle, we sought a new method to monitor retinal degeneration. During retinal degeneration, damaged cells might release proteins into surrounding tissues, but have gone undetected due to the invasive nature of retinal biopsy procedures. We hypothesized that proteomic analysis of the adjacent vitreous may serve as way to indirectly biopsy the retina during early degeneration and identify changes in the retinal proteome.

Methods
To identify potential vitreous biomarkers for retinal degeneration, we studied protein expression in the retina and vitreous of the PDE6-/- mouse, an animal model for rapid retinal degeneration. Tandem liquid chromatography mass spectrometry (LC-MS/MS) was performed on retina and vitreous samples in wild-type and PDE6-/- mice during early, middle and late stage disease. Vitreous proteins were analyzed using 1-way ANOVA followed by unbiased heatmap clustering.

Results
We identified a number of retinal proteins that migrated into the vitreous of degenerating eyes. Interestingly, there were 119 retinal proteins that were present in the vitreous of the PDE6-/- mouse on day 15, much earlier than established physiological biomarkers of vision loss. These proteins were further analyzed using gene ontology, pathway, and network analysis to identify perturbed protein pathways during early retinal degeneration. Some of these candidate biomarkers were then validated by LC-MS/MS analysis on a vitreous biopsy sample from a patient harboring the same disease mutation.

Conclusions
Proteomic profiling of vitreous biopsies is far less invasive than retinal biopsy and may help to identify and monitor patients at risk for retinal degenerative diseases.

Keywords
Retinal degeneration, vitreous biopsy, biomarkers, PDE6

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Phosphoproteomic analysis of acquired resistance to trastuzumab and dual HER2-blockade in HER2-positive breast cancer cells

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Background
While HER2-overexpressing breast cancers targeted by trastuzumab frequently acquire resistance to this therapy, dual HER2-blockade with trastuzumab and pertuzumab significantly improves the prognosis of this disease. In this study, we used an integrative approach to compare global protein expression and tyrosine phosphorylation events involved in both acquired trastuzumab-resistance and response to dual HER2-targeted therapy with trastuzumab and pertuzumab.
Methods
We performed phosphotyrosine peptide immunoprecipitation coupled to quantitative label-free mass spectrometry and database searching in an established in vitro model of acquired trastuzumab-resistance to investigate initial sensitivity to trastuzumab, eventual reprogramming in resistance and inhibitor response to dual therapy. Dedicated computational approaches were combined to identify altered signalling networks and molecular reprogramming upon trastuzumab-resistance.
Results
In total, our study yielded 2457 phosphopeptides on 1134 proteins, including 1855 class I phosphopeptides and 94 protein kinases. Compared to baseline, incubation with trastuzumab and dual therapy of trastuzumab-resistant cells resulted in downregulation of 218 and 356 phosphopeptides, respectively, while 294 and 218 phosphopeptides were upregulated (Fc> 2). Interestingly, exposure of trastuzumab-resistant cells to dual therapy resulted in 24 phosphoproteins significantly more downregulated than compared to exposure to trastuzumab. We further prioritized certain signalling nodes by overlapping: (i) differential expression in untreated resistant and parental cells, (ii) regulation relevant for sensitivity in response to both trastuzumab and dual therapy of parental cells, (iii) opposite/deficient regulation in response to trastuzumab of resistant cells, and (iv) same response to dual therapy of resistant and sensitive cells.
Conclusions
Our data confirmed previously known roles for Ras/MAPK and PI3-kinase signalling in trastuzumab-resistance, as tyrosine-phosphorylated FGFR1, FGFR3-4, MET, MAPK3, and PIK3R1-3 were upregulated in trastuzumab-resistant cells. Altogether, our phosphoproteomics study provides network-level insights into molecular alterations associated with cancer drug resistance and suggests potential biomarkers and treatment options for trastuzumab-resistant tumors.
Keywords
Phosphoproteomics, HER2-positive breast cancer, Resistance, Pertuzumab, Trastuzumab

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Single Amino Acid Resolution of Glycosites Using Top-down UVPD of Glycoproteins

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Background

Ultraviolet photodissociation (UVPD) is a powerful tool for top-down proteomics due to the high efficiency and indiscriminant nature of its fragmentation. While UVPD has been demonstrated for glycopeptide and glycan analysis, it has not yet been tested on intact glycoproteins. Here we demonstrate the utility of top-down UVPD for analyzing both the composition and locations of glycosylations.

Methods

Disulfide intact and reduced and alkylated glycoprotein ions were produced by static nano-electrospray ionization from denaturing solutions of 49/50/1 water/methanol/acetic acid. Ions were analyzed on a Thermo Orbitrap Fusion Lumos Tribrid MS, and top-down fragmentation was performed using HCD, ETD, or ultraviolet photodissociation at 213 nm for each ion.

Results

UVPD fragmentation of disulfide reduced proteins produced predominantly a- and x-ions with preferential fragmentation at proline residues, consistent with previous observations for glycopeptides. UVPD of disulfide reduced ribonuclease B resulted in single amino acid resolution for the site of the glycan, and fragment ions were composed predominantly of cleavage along the protein backbone with retention of the entire glycan. Whereas UVPD of disulfide intact ribonuclease B resulted in predominantly cleavage of the entire glycan from the precursor and charge reduced precursor ions, with close to 100% sequence coverage for the termini of the proteins up until the locations of the disulfide bonds. This data demonstrates that complementary information can be gained from top-down UVPD of folded and unfolded glycoproteins; the exact masses of the glycans from UVPD of the folded form of the protein, and the residues on which those glycans are located from UVPD of the unfolded form of the protein.

Conclusion

This is the first demonstration of the power of UVPD for top-down analysis of glycoproteins.

Keywords

Top Down, UVPD, Proteomics

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In-depth quantitative mass spectrometry reveals the region and cell-type resolved proteome of the human heart

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Background

Proteomic atlases, such as mouse liver and brain have recently been established and are widely used. The human heart is very important from a medical point of view, as heart diseases remain the leading cause of death. The heart tissue is very difficult to analyze by proteomics since it is dominated by a small number of proteins involved in the contractile apparatus. This has confined previous analyses to a few hundred or a few thousand different proteins. In this study, we determine the healthy human heart proteome by high-resolution mass spectrometry (MS) based proteomics.

Methods

We applied ''in-StageTip'' sample preparation to three healthy adult hearts divided into 16 regions and four major cardiac cell types. After tryptic digestion, we used high pH reversed-phase fractionation. The recently described loss-less spider fractionator (PMID: 28126900) enabled efficient fractionation of a total of only 30µg of peptides into 8 fractions. Applying a state of the art label free LC-MS/MS workflow, we measured 400 runs by shotgun proteomics on a QExactive HF mass spectrometer.

Results

We quantified over 11,000 proteins from this challenging tissue and showed that we can differentiate heart areas based on proteomics data. We distinguished protein isoform expression in many cases and quantified the subcellular distribution of the heart proteome in terms of copy numbers per cell. IIllustrating the usefulness of this resource, we further showed that our heart atlas can be applied to define molecular differences between healthy and diseased tissue samples. We showed for the first time that patients suffering from atrial fibrillation show distinct molecular signatures and that our proteomics workflow in combination with technological advances can be easily implemented for the analysis of any heart sample.

Conclusion

We provide a rich proteomics resource for analyses of normal heart function and disease.

Keywords: Mass spectrometry, Proteomics, Human Heart

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Identification of tear fluid biomarkers in dry eye and glaucoma patients using targeted proteomics strategy

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Dry eye syndrome (DES) and glaucoma are debilitating ocular pathologies that affect a large population, yet the fundamental etiology underlying these diseases remains elusive. Moreover, biomarker discovery in the tears of DES and glaucoma patients is still limited, which deters development of improved diagnostic and prognostic platforms. Hence, this study endeavored to investigate specific proteome alterations in individual tear samples of DES and glaucoma patients.

Tear samples were collected using Schirmer's strips from 72 patients subdivided into glaucoma (GLM; N=18), aqueous-deficient dry eye (DRY; N=18), a combination of the two diseases (GLM_DRY; N=18) and healthy subjects (CTRL; N=18). Samples were analyzed individually employing a targeted mass spectrometry (MS) strategy called accurate inclusion mass screening (AIMS). The acquired continuum MS spectra were analyzed by MaxQuant computational proteomics platform followed by functional annotation and pathway analyses.

A total of 64 signature peptides representing various tear protein isoforms were characterized and utilized for the targeted MS analysis. Among these, 30, 23 and 37 tear proteins were significantly ($P < 0.05$) differentially expressed in the GLM, DRY and GLM_DRY groups, respectively. A large majority of the differentially expressed proteins are involved in inflammatory, metabolic and apoptotic processes. Differential expressions of certain clusters of proteins were exclusive to a particular disease group, namely IGHA1, PIGR, and C3 in GLM and, SCGB1D1, ALDH3A1 and ANXA2 in DRY. Importantly, there are inter-individual variations in the expression levels of specific protein isoforms.

This study demonstrates that the targeted MS strategy is instrumental for unambiguous identification and quantification of specific protein isoforms in individual tear samples of DES and glaucoma. Multiplexed measurements based on the signature peptides via targeted strategy have the potential to provide invaluable hints on development of specific diagnostic and prognostic tools for both ocular pathologies in larger cohorts.

Tears, Dry eye, Glaucoma, Targeted proteomics

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Profiling basement membrane proteins in plasma and cerebrospinal fluid within ALS and FTD

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Background

Muscle weakness and atrophy as a result of motor neuron degradation are the primary and most prominent symptoms of amyotrophic lateral sclerosis (ALS). Several recent studies also indicate that a vascular disease component is present among the ALS symptoms. Both the blood-brain barrier and the blood-spinal cord barrier are damaged in patients with ALS and transgenic mouse models show signs of disruption prior to disease onset. To investigate the connection to vascular disruption we profiled about 100 basement membrane proteins in ALS patient plasma. We further extended our study to profile cerebrospinal fluid from a smaller set of frontotemporal dementia (FTD) patients. Although ALS and FTD are heterogeneous at the clinical and neuropathological level they share several features, not least abnormal aggregation of the TDP-43 protein. Comparing patient samples from the two disease is therefore highly relevant to further investigate the connection between them.

Methods

By directly labeling the samples with biotin and coupling antibodies to magnetic color-coded beads, more than 1 200 samples from Belgium, Germany, the Netherlands, Poland, Sweden and the US could be profiled. These included both patients and healthy controls and relative protein amounts were detected in a flow cytometry-like system by addition of a streptavidin-coupled fluorophore.

Results

Apart from straight forward case-control comparisons the clinical data connected to the samples will enable us to study protein levels in association to age at onset, disease duration and progression rate. By including FTD patients in our study we wish to a) evaluate if the measured proteins could have a potential relevance for FTD and b) attempt to determine if the observed protein patterns are ALS-specific or overlapping between the two diseases.

Conclusions

Our aim is to better understand if and how basement membrane disruption contributes to ALS pathology, and map the potential protein overlap with FTD.

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Breast cancer classification based on proteotypes obtained by SWATH-MS

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Background: Accurate breast cancer classification is vital for patient management decisions and better tumor classification is expected to enable more precise and eventually personalized treatment to improve patient outcomes. However, despite advances in classification of the malignancy, current therapies fail a substantial proportion of patients.
Methods: Here, we present a novel quantitative proteotyping approach based on SWATH mass spectrometry and a multigroup classifier for breast tumor classification derived from proteotype data. The study was based on 96 tissue samples representing five breast cancer groups as classified by their immunophenotype. Independent sample sets were involved to validate the findings at protein and transcript level.
Results: To extract quantitative protein information from SWATH-MS datasets, we firstly generated a breast cancer specific spectral library based on reference spectra for 28,233 proteotypic peptides (FDR<0.01), representing 4,443 proteins. Correlation of extracted proteotype patterns for 96 individually measured patients indicated groups that largely recapitulate the immunophenotypes. However, the proteotype-based classification also revealed varying degrees of intergroup heterogeneity with triple negative tumors being the most heterogeneous. We identified three proteins that contributed most strongly to the separation of the groups, which are associated with estrogen receptor status, tumor grade and HER2 status, respectively. Three key classifiers exhibited high levels of correlation between protein and transcript levels (R>0.67), while general correlation did not exceed R<0.29. Hence, the strongest protein markers in breast cancer, including our classifier proteins, appear to be driven by transcriptional regulation, whereas the majority of proteins differentially expressed are regulated by alternative mechanisms.
Conclusions: Our data indicate how large-scale protein-level measurements by next-generation proteomics can improve tumor classification and potentially lead to better patient stratification for precision medicine.
Keywords: breast cancer, SWATH-MS, proteomics, transcriptomics

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Phosphoproteome networks identify focal adhesion kinase as new target in pancreatic ductal adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal cancers. The poor prognosis warrants new strategies to understand the aggressive biology and identify new drug targets. Unbiased discovery mass spectrometry (MS/MS) based phosphoproteomics can function as a screening for targets, since it highlights key aberrantly activated kinases and pathways important for this tumor. In this study, we employ phosphotyrosine(pTyr)-based phosphoproteomics on preclinical PDAC models and patient tumors to unravel new drug targets.

Methods

We performed phosphoproteomics on 11 PDAC cell lines, 7 primary cell cultures and 16 fresh frozen human tumors. Lysates were treated with phosphatase inhibitors and digested with trypsin. pTyr-peptides were enriched and analyzed by high-resolution nano-LC mass spectrometry.

Results

We identified 138 different phosphorylated kinases with our pTyr workflow. Pearson correlation coefficients showed excellent reproducibility for technical ($r = 0.937$) and biological ($r = 0.876$) replicates. In our cell line panels, multiple kinases and their downstream targets were commonly highly phosphorylated. Focal adhesion kinase (FAK) was identified in multiple (primary) cell lines. Pharmacological inhibition with defactinib proved FAK as a possible anti-proliferative target in PDAC. Moreover, inhibition resulted in significant reduction of the migratory potential (p-value < 0.001). Combination treatment with chemotherapeutic paclitaxel resulted in synergistic lethality. Additionally, activated FAK was identified in fresh frozen tumors, validating our in vitro models.

Conclusions

Our p-Tyr phosphoproteome screening of PDAC revealed high phosphorylation levels of multiple kinases. The abundancy of highly active tyrosine kinases may be correlated to the aggressive biology of this disease. We have shown that this approach can identify new targets in vitro. Combination therapy of paclitaxel with FAK inhibition might be a new drug regimen for PDAC patients. This study prompts further validation and prognostic evaluation of the identified active kinases to improve treatment of PDAC.

Keywords
Phosphoproteomics, PDAC, focal adhesion kinase, paclitaxel

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Affinity proteomic profiling of plasma for proteins associated to pancreatic cancer

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Background

Pancreatic cancer (PAC) is the fourth leading cause of cancer-related deaths in the Western world, and less than 5% of PAC patients survive after five years. Here, we investigated the plasma proteomes of PAC patients using a multiplexed antibody bead array with the aim to identify proteins that are organ-specific and associated to PAC. The candidate proteins could complement existing tools for early detection of PAC, assessment of tumor stages, and predictors of patient survival.

Methods

With a resource of antibodies provided by the Human Protein Atlas, we have developed a bead-based antibody assay enabling high-throughput screening and protein profiling of biofluids. A set of 64 plasma samples from the EndoTAG study was analyzed with 10,261 antibodies. In order to confirm initial findings, a second independent set of 399 plasma samples from the BIOPAC study was profiled, comprising of 186 patients with stage 1-3 and 189 stage 4 tumors. A targeted bead array comprised of 379 antibodies was assembled including antibodies towards tumor-stage associated proteins, targets based on RNAseq annotated as enriched in the pancreas, and targets listed in the Pancreatic Cancer Database.

Results

Among several candidates associated to cancer stage (243 out of 10,261), we also found proteins linked to survival (p-value <0.001). Antibody performance and on-target binding has been validated by immunocapture mass-spectrometry for the development of targeted dual-binder immunoassays.

Conclusions

By employing a multiplexed affinity proteomics approach, we have investigated two study sets for pancreas-specific proteins in plasma associated with PAC. Antibody performance has been validated by immunocapture mass-spectrometry for the development of targeted immunoassays that could be used in larger, independent longitudinal studies. Upon further evaluation, the current findings may serve as a potential complementing tool for prognosis and monitoring of tumor progression in PAC.

Keywords

Affinity proteomics, plasma profiling, pancreatic cancer

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New Signaling pathways altered in Restrictive Cardiomyopathy revealed by Tandem Mass Tag proteomics and phosphoproteomics

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Background

Heart disease is a major cause of death worldwide. One type of heart disease associated with a high incidence of sudden cardiac death is restrictive cardiomyopathy (RCM). RCM, associated with the inability of the ventricles to relax normally, is the least understood of the three major forms of cardiomyopathy, and little is known about how mutations in proteins cause RCM.

Methods

We investigated an R145W cardiac troponin I (cTnI) transgenic mouse model of RCM using Tandem Mass Tag (TMT) proteomics and metabolomics to determine the key pathways involved in the cardiac dysfunction. TMT proteomics of total heart lysate from wild-type and R145W hearts as well as separate TMT phosphoproteomics of wild-type and R145W hearts were carried out (n=4 for each sample). Subsequent studies using western blotting and several biological assays were utilized to investigate the signaling pathways involved.

Results

Several pathways were found to be affected in the R145W RCM hearts, including homeostatic, metabolic, developmental, cell communication, and apoptosis pathways. Several proteins that are part of the ubiquitin-proteasome system (UPS) were differentially expressed between R145W and WT hearts. Investigation of the UPS, which is important in maintaining cellular homeostasis, showed that 3-month-old R145W hearts had lower proteasome activity (proteasome dysfunction) compared to wild-type hearts. Metabolomic experiments showed significant changes in several metabolites important in energy production and levels of ATP were decreased in R145W hearts relative to WT hearts. Phosphoproteomic analysis showed 103 phosphorylation sites were differentially expressed with changes in the phosphorylation sites on several myofilament proteins such as titin and regulatory light chain.

Conclusions

Besides energy metabolism, which seems to be altered in all cardiomyopathy models investigated, the UPS and altered myofilament phosphorylation levels are novel pathways that are significantly affected in R145W RCM hearts.

Keywords
restrictive cardiomyopathy; Tandem mass tags; ubiquitin-proteasome system

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Analytica: software for LC-MS omics data analysis and visualisation

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Background
Analysing data generated from LC-MS datasets often requires access to an informatician or use of scripting languages to understand the nature of an experiment or produce quality graphics. The need to quickly and intuitively understand data translates to a need for software meeting such requirements, allowing bench scientists that capability. We are developing an informatics tool, currently called 'Analytica' to provide quantitative/qualitative visualisations for omics LC-MS data in a variety of formats, allowing a standardised view/analysis of a specific experiment type, regardless of where/how the data were produced.

Methods
The Analytica software is being developed in partnership with industry and academia. The software is being developed mainly as a Java(FX) application using IntelliJ Idea, SceneBuilder and JIRA development software. New visualisations are added to the desktop version and the command line version, while another component handles data reading. Feature requests and feedback are taken from external collaborators and from research groups to enrich the application for its target users.

Results
Visualisations have been developed for text-based processed label-free and SILAC formats. These include volcano, abundance/ratio, and various clustering plots and more for label-free/SILAC formats. In addition to protein data, peptide and small molecule data are supported. Interactivity of the software is an important feature - plots have been developed to be searchable/zoomable; axes changeable; datapoints malleable. Plots can be exported in a range of formats. The command-line version allows integration with third-party workflows (e.g. Symphony) and is being evaluated for QC purposes where when acquisition is completed, downstream analysis begins resulting in QC data visualisations being generated. Capabilities are under development for performing computational biology analyses, including pathway mapping and gene set enrichment analysis.

Conclusions
Analytica enables quick visualisation of experiments without requiring informatics skills, and supports an expanding list of formats with rich interactivity.

Keywords
Visualisation Proteomics Progenesis

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A clinical proteomics success story: from proteomics-based biomarker discovery to clinically actionable test

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Background
In recent years, improvements in both mass spectrometer design and bioinformatics approaches have contributed to significant advancements in the field of proteomics.
However, success in translating proteomics to a clinical test is limited due to a myriad of challenges (i.e. scientific, regulatory, reimbursement, etc.). We provide here a success story in translating a discovery proteomics signature for the second deadliest cancer in the United States, Colorectal Cancer (CRC), into a commercially offered clinical test.

Methods

A curated list of >300 proteins related to CRC from literature and public databases was developed into a MRM assay. Tier 2 assays were developed for all 187 proteins and incorporated into a single 30 minute MRM assay.

From the validated MRM based classifiers, 27 immunoassays were developed on a multiplexed platform. The development of these immunoassays encompassed multiple phases including feasibility, development and optimization. All assay panels were fully automated and evaluated for accuracy, precision, selectivity and specificity.

Results

The MRM assay of 187 proteins was used to evaluate 274 individual patients, which resulted in multiple validated classifiers (Max AUC of 0.91). After taking all aspects of commercialization into consideration, multiplexed immunoassays were developed for 27 proteins from the validated classifiers.

The 27 immunoassays, multiplexed into 5 panels, were then used to evaluate 4435 individual patients from a new sample cohort. Resulting validated CRC and AA classifiers, comprised of 15 proteins, from a 1336 holdout sample set showed a CRC AUC = 0.86 and AA AUC = 0.69. This signature was then developed to all CLIA & CAP requirements for quantitative clinical chemistry assays and made commercially available in 49 US states.

Conclusions

While the translation of discovery proteomics to commercially available clinical tests remains challenging, rigorous study design and assay development increase the probability of success.

Keywords

Clinical

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Proteome-wide profiling of exosomes revealed exosome-driven intercellular communications in gastric cancer microenvironment and macroenvironment

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Introduction

Extracellular vesicles (exosomes) play various roles in mutual communications between cancer cells and extracellular environment. To understand the significance of exosome-mediated protein transportation in cancer development or progression, we developed a high-purity exosome isolation tool (EV-Second columns) and performed proteome-wide quantitative profiling of serum exosomes derived from gastric cancer (GC) patients or healthy donors.

Methods

Serum samples were collected from 10 healthy donors and 48 GC patients. Following exosome isolation by EV-Second columns based on mixed modes of size exclusion and weak hydrophobic interaction, exosomal proteins were subjected to LC/MS analysis. Protein identification, label-free quantification, and subsequent statistical analysis were performed by MaxQuant. Proteins specifically detected in GC-derived exosomes were functionally evaluated.

Results

The LC/MS analysis identified 822 exosomal proteins in which 13 proteins showed significant up-regulation in GC patients' exosomes (t-test, $p < 0.05$, fold change > 2.0). Among them, frequent overexpression of PN-1 protein in GC cells (80.0% of undifferentiated carcinoma or 59.1% of adenocarcinoma) was confirmed by multiple tissue array analysis (n = 327). Interestingly, incorporation of PN-1++ exosomes drastically prevented anoikis of the recipient cells. Further single cell pH reporter assay revealed that PN-1 enzyme inhibited pre-apoptotic intracellular pH change, leading to survival of cancer cells.
CagA, a pathogenic factor of H. pylori, was also found in serum exosomes from GC patients (Sci. Rep., 6:18346, 2016). CagA in GC cell-derived exosome was efficiently transferred into recipient cells and induced typical morphological change, indicating that H. pylori proteins were transported exosomes in blood circulation and may be involved in cancer development and also extragastric diseases. Indeed, H. pylori infection increases incidence of non-gastrointestinal diseases such as cardiovascular diseases.

Conclusion

These data suggested that cancer-related exosomes are served as key mediators controlling both tumor microenvironment and macroenvironment, which could provide novel mechanisms underlying tumor development or progression.

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EPIQ (Epic Protein Integrative Quantification): Ultra-sensitive n-plexed isotopic labeling-based quantification by a model-based reconstruction method

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Background
Isotopic-labeling based protein quantification has advantages such as accurate quantity ratios and reduced technical bias over other approaches. However, conventional isotopic-labeling schemes (e.g., SILAC) have a limited multiplexity (≤3-plex). Although some trials have been made to increase multiplexity, they either necessitate expensive resources or lack dedicated analysis tools. Also, thorough evaluation of quantification was scarcely made or the number of proteins quantified in all labels was often insufficient for most applications.

Methods
EPIQ allows deuterium-based isotopic-labeling and small mass difference between labels (≥2Da). Such labels make the XICs (eXtracted-Ion Chromatograms) from distinct labels hard to be separated; they have different retention time (deuterium effect) and mutual interference (isotope). To address these hindrances, EPIQ uses a model that assumes how such XICs are generated. Given observation, EPIQ predicts retention time shifts, isotope distribution, and XIC shapes that constitute the model. Then it reconstructs the observation, successfully separating XICs from distinct labels for accurate and sensitive quantification.

Results
We developed deuterium-based 6-plexed labeling. Labeled HeLa unfractionated sample having ratio 30:20:10:1:5:10 was subject to LC-MS/MS (Q-Exactive). EPIQ reported ~2,500 proteins with median quantity ratio 29.5:20:9.9:1.3:4.3:9.5. In ~77% of the cases, the ratios (to the first label) fell within 2-fold change from the input ratio.
To benchmark against other state-of-the-art tools, we adopted 13C-based 3-plexed labeling. A sample with a known ratio (HeLa, 1:10:20) and a biological sample (Xenopus early embryo) were analyzed by EPIQ and other tools. EPIQ reported 1.2-1.5 times more proteins and showed less compromised ratios even for low-abundant proteins than other tools.

Conclusions
EPIQ enables ultra-sensitive n-plexed quantification without requiring complicated/expensive labeling or high-resolution mass-spectrometry. As EPIQ allows higher multiplexity, we are currently developing further chemical/metabolic labeling schemes (≥8-plex). EPIQ could facilitate various biological applications (e.g., cell dynamics studies or sensitive detection of differentially expressed proteins).

Keywords:isotopic-labeling;protein quantification;deuterium effect

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HIV Latency: Deep-proteome coverage reveals the extent of cellular awareness of HIV and immunotherapy targets

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Background
HIV latency is the biggest hurdle in eradicating the virus. Understanding the differences in protein expression during latency and early stages of reactivation could help define the mechanisms that maintain HIV persistence and provide targets to eliminate latently-infected cells. While several mRNA expression studies exist, there is evidence that analysis of protein products can provide orthogonal information. To address this, a well-established latently-infected T-cell line derived from Jurkat cells (J-Lat) has been used in what we believe is a first comprehensive study exploring global proteomic changes during latency and after reactivation by an external stimulus such as TNF-α.
Methods
Latently infected J-Lat cells (clones 6.3 and 10.6) and parental Jurkat cells (n=3) were cultured with or without TNF-α. Cell lysates were reduced, alkylated, and digested with trypsin, fractionated offline and analysed by Q-Exactive mass spectrometer. Data was analysed using MaxQuant and Perseus.
Results
More than 9000 proteins were identified and 7500 proteins quantitated in latent and reactivated cells, making this study the largest of its kind. Several pathways were perturbed in latently-infected cells including proteins involved in cell signalling, energy generation, and key transcription factors. Several host viral-restriction factors especially IFN-γ inducible proteins were upregulated during latency and abrogated upon activation of the virus by TNF-α. Additionally, 22 cell surface proteins including previously described protein, CD2, were also upregulated during latency.
Conclusions
The proteomic data points to cellular awareness of the latent virus as demonstrated by upregulation of key antiviral response pathways, host-restriction factors and deprivation of host-factors necessary for HIV replication. Surface-expressed proteins up-regulated could serve as markers for identification and targeting of latently-infected cells. This study has developed a large database of dysregulated proteins that can be interrogated to yield information on the expression level of any of the 9000 proteins identified.
Keywords
HIV, latency

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Identification of non-invasive Urinary novel biomarkers in Urothelial carcinoma of bladder: A Proteomic approach

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Background:
Bladder cancer is 9th leading cause of cancer with 430,000 new cases and 165,000 deaths occurring in 2012. Urine represents an ideal source of clinically relevant biomarkers as it contains a large number of proteins and low molecular weight peptides. The survival rate is better when the disease is detected early. The comprehensive characterization of the urinary proteome in urinary bladder cancer can serve as a reference for non-invasive biomarker discovery. Current diagnostic biomarkers are not sufficiently sensitive for substituting or complementing invasive cystoscopy.

Methods:
A 50 ml urine sample was collected from bladder cancer patients and healthy controls. We employed 2D Gel electrophoresis to separate proteins. 20 proteins were differentially expressed in patient's vs healthy controls, which were then analyzed using MALDI TOF/MS. We have also validated the mRNA expression of identified proteins in urine by Q-PCR. Tissue sections from urothelium of bladder cancer patients along with adjacent non-tumor sections were further validated at mRNA and protein level using Q-PCR and Western Blotting, respectively.

Results:
Out of differently expressed proteins analyzed in 20 spots, 3 proteins (A, B and C) were chosen since these were reported to be involved in other cancers. These molecules were further found to be over-expressed in tumor tissues as compared to adjacent non-tumor tissue at mRNA and protein level both as well as mRNA level in Urine.

Conclusions:
Identified proteins have an established role in other cancers and we have also observed increased expression in bladder cancer. Overall, Protein A showed most significant expression during MALDI TOF/MS analysis and upon further validation at mRNA and protein levels in tissues thus represents potential biomarker for noninvasive screening for bladder cancer. These proteins might become valuable target for cancer therapy in future and potential diagnostic tool in bladder cancer.

Keywords: Proteomics; Urine; Bladder Cancer; Biomarkers

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Highly sensitive quantitative phosphoproteomics and primary-sequence-based scoring reveals putative substrates of ERK.

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Background
Recent advances in LC-MS together with phosphopeptide enrichment techniques have allowed the identification of thousands of phosphopeptides from complex biological samples. However, it is still hard to achieve deep and high throughput quantitation in phosphoproteomics for a small sample amount. Therefore, the development of novel method for high sensitive and high throughput phosphoproteomics is required. In this study, we developed a micro-scale isotope tagging coupled with high resolution LC-MS using a meter-long monolithic silica column for deep and high throughput quantitation, and applied it to the HeLa phosphoproteome change upon MKK1/2 inhibitor treatment.
Methods
HeLa cells were treated with selumetinib or vehicle DMSO. Tryptic digests from HeLa lysates (50 µg) were used for phosphopeptide enrichment by titanium dioxide chromatography with lactic acid. The enriched phosphopeptides were labeled with TMT according to a new labeling protocol using solid-phase extraction tips as microreactors. Multiplex TMT samples were mixed equally and measured by one-shot LC-MS using a 2-meter long C18 monolithic silica capillary column. The TMT ratios were combined with primary sequence preference (PSP) score of ERK1 to identify putative ERK substrates.
Results
We first examined the solid-phase TMT reaction with small sample amounts and found that hydrophobic polymer beads together with ion pair reagents at neutral pH gave the best results among the published protocols in terms of the number of identified phosphopeptides. We successfully quantified 10,030 phosphopeptides from triplicate LC-MS runs. Based on TMT ratios and PSP score, we identified 123 putative ERK substrates including NUP50_HUMAN, a known in vivo substrate of ERK1.
Conclusions
We developed a novel strategy for high-throughput and micro-scale phosphoproteome analysis. This method is applicable to phosphoproteome profiling of scarce samples.
Keywords
Phosphoproteomics, solid phase TMT labeling, kinase substrate

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Proteomics as part of systems toxicological assessment of a mentholated candidate modified risk tobacco product

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Background
Modified risk tobacco products (MRTPs) are being developed with the aim of reducing smoking-related health risks. The Tobacco Heating System 2.2 (THS2.2) is a candidate MRTP that uses the heat-not-burn principle. Systems toxicology approaches were engaged to assess the respiratory effects of mentholated THS2.2 (THS2.2M) in a 90-day rat inhalation study (OECD test guideline 413).
Materials & Methods
The analyses of respiratory nasal epithelium and lung tissues were performed using quantitative iTRAQ approaches. 6 rats per groups were nose-only exposed to filtered air (Sham), reference Cigarette Smoke (CS) or to three concentrations of THS2.2M aerosol. 6 females were also exposed to Sham, CS, and two concentrations of THS2.2 M and were kept for a 42 days recovery period.
Results
The adaptive response of the respiratory nasal epithelium to conventional cigarette smoke (CS) included squamous cell metaplasia and an inflammatory response, with high correspondence between the molecular and histopathological results. In contrast to CS exposure, the adaptive tissue and molecular changes to THS2.2M aerosol exposure were much weaker and were limited mostly to the highest THS2.2M concentration in female rats. In the lung, CS exposure induced an inflammatory response, triggered cellular stress responses, and affected sphingolipid metabolism. These responses were not observed or were much lower after THS2.2M aerosol exposure.
Conclusions
Overall, this system toxicology analysis complements and reconfirms the results from classical toxicological endpoints as well as the transcriptomics and lipidomics endpoints. This results further suggests potentially reduced health risks of THS2.2M.
Keywords
Modified risk tobacco product, Systems toxicology, proteomics
References
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Monitoring kinase activity using customized substrate peptides

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Background
Protein kinase is one of the key components in phosphorylation-based signal transduction, and aberrant kinase activity often results in severe diseases such as cancer, rheumatism, and CNS disorders. Therefore, quantifying kinase activities is valuable for diagnostic and therapeutic purposes. While recent advances in phosphoproteomics enable us to identify thousands of phosphosites, it is still challenging to obtain kinase activity profiles because the information of kinase-substrate relationship is mostly missing. In this study, we designed specific substrate peptides for each kinase.

Methods
To select the amino acid sequence of kinase-specific substrate peptides, in vitro reactions using 398 recombinant human kinases and HeLa cell extracts were performed and phosphorylated sites were determined by quantitative phosphoproteomics approach using nanoLC-MS/MS. Based on the sequence information of these substrates, the specific and sensitive sequences for each kinase were predicted, synthesized and experimentally confirmed under in vitro and in vivo conditions using recombinant and endogenous kinases in cells, respectively. Quantitation of the phosphorylated form of customized substrate peptides was carried out using nanoLC-MS/MS.

Results
We successfully designed the substrate peptides for each kinase or kinase family both with high kinase selectivity and high sensitivity under the in vitro condition. We also confirmed that these customized peptides can be used to monitor the intrinsic kinase activity fluctuated by activators and/or inhibitors treated in cells. Furthermore, we observed that the customized substrate peptides in the presence of a cell penetration assisting peptide were successfully phosphorylated in living HeLa cells.

Conclusion
We customized kinase-specific substrates and applied to monitoring kinase activity in cells.

Keywords
protein kinase, phosphorylation, signal transduction

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Quantitative phosphoproteomic profiling to identify new drug targets to treat chronic obstructive pulmonary disease

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Chronic obstructive pulmonary disease (COPD) is the 3rd most common cause of death worldwide, with 50% of patients succumbing to the disease within 10 years of diagnosis. Currently, there is no cure for COPD, only ineffective remedies that reduce its symptoms. Our laboratory has developed a unique and clinically relevant mouse model of cigarette smoke-induced COPD that recapitulates the hallmark features of the human disease in 8 weeks. Using this powerful discovery research tool coupled to comparative and quantitative phosphoproteomic profiling has facilitated the assessment of signalling pathways regulated by chronic exposure to cigarette smoke. Mice were exposed to a smoking regimen for 4, 6, 8 and 12 weeks, and compared to age matched controls (room air). We have assayed changes in the activity of signalling proteins associated with cigarette smoke exposure in the lungs of these mice by employing a multidimensional phosphopeptide enrichment strategy coupled to high-resolution quantitative proteomics using isobaric tags. We have progressively tracked 2,000 phosphorylated proteins over the course of the exposure and quantified over 15,000 phosphopeptides. In addition to this, our strategy has identified over 4,500 unmodified proteins and 1,500 glycosylated proteins. Network signalling pathways analysis on phosphopeptides changes in the lungs of mice revealed important modulation of structural proteins associated with airway remodelling known in the pathobiology of COPD. Changes in the phosphoregulation of the actin cytoskeleton, tissue developmental processes and cellular survival pathways were also observed. These analyses are providing us with the capacity to progressively track changes associated with chronic exposure to cigarette smoke and will help improve our understanding of human COPD, and ultimately lead to more meaningful treatment options. Future directions will see the validation of these signalling pathway changes in relevant human tissues and assessment of the therapeutic efficacy of novel COPD drug targets in our mouse model.

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Accurate LC retention time prediction for metabolites using calibrated predictions

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Accurate prediction of liquid chromatographic retention times of molecules is useful for better identification in untargeted MS and limiting experimental measurements in targeted MS. In ref. [1] retention time predictions were used in an untargeted MS experiment of a complex sample to identify the correct lipids that have multiple isobaric candidates. However, different experimental setups (differences in columns, gradients, solvents, stationary phase, etc.) gave rise to a multitude of prediction models that only predict accurate retention times for a specific experimental setup. In practice this typically results in fitting a new predictive model for each new experimental setup (even just for a new column).

In this research we introduce the concept of generalized calibration that builds on the database controlled calibration approach implemented in PredRet [2]. Our key idea is to fit calibration curves on predicted retention times instead of limiting ourselves to observed retention times only. Machine learning models are fitted to specific experimental setups and calibrated predictions for an experimental setup of interest are used to fit a predictive model for this setup. As PredRet is limited by predicting retention times only for the molecules that are in the database, our approach is able to accurately predict the retention time of any molecule.

We show that our approach results in significantly higher accuracy of predicting the elution peak than experimental setup specific models individually. When compared to a published model for lipids [1] a higher performance in the mean absolute error of 25 % is observed when using generalized calibrations. For the same dataset the mean absolute error for predicting the elution peak with 100 training examples is 8 seconds.

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Universal solid-phase protein preparation (USP3) for bottom-up and top-down proteomics

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Background
Traditional bottom-up MS-based proteomics strategies rely on multi-step sample preparation workflows to enable the comprehensive analysis of the proteome via LC-MS. Examples include the membrane-based methods (FASP), suspension trapping methods (S-TRAP) and on-bead digestion protocols, namely the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3). We present an optimised on-bead digestion protocol (USP3) that builds on the original SP3 workflow, which not only allows for enhanced protein precipitation, but for the first time, enables a robust sample preparation workflow for top-down MS.

Methods
We compared various buffers for precipitating protein onto magnetic SP3 beads. Once the buffer conditions were optimised, we proceeded to evaluate the digestion efficiency using 100 µg HeLa cell lysate. We benchmarked USP3 against current methods including FASP, S-TRAP and the SP3 method. Bottom-up MS analyses were performed on an Impact II UHR-QqTOF MS (Bruker). For intact MS, we used the USP3 method to prepare 50 µg HeLa cell lysate (5 replicates). Eluted material was analysed on the MaXis II UHR-QqTOF MS (Bruker).

Results
Bottom-up MS analyses revealed the USP3 (1 hr precipitation/1 hr digestion) and FASP methods out performed all other methods in terms of the number of unique protein and peptide identifications. We observed the most enzyme miscleavage events in the S-TRAP method with <20% of peptides with 1 or more miscleavage. In contrast, both the 1 hr USP3 and FASP methods had comparable levels of 1 or more miscleavage events (<12%). Overall, the 1hr USP3 method had reduced technical variation across replicate analyses.

Conclusions
Here we describe universal, solid-phase protein preparation (USP3), a robust and scalable method which provides an efficient means of protein clean-up for intact protein profiling. It combines the advantages using detergent-based protein solubilisation methods with a fast, economical and highly-reproducible method for routine protein digestion.

Keywords: on-bead digestion, intact MS, protein precipitation

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Suppression of colorectal cancer proliferation and invasion by antagonising uPAR·αvβ6 interaction

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Background

Colorectal cancer (CRC) metastasize to local lymph nodes or distal organs at its later stages, decreasing survival rate . Currently, late clinical stage treatment options are almost non-existent or are highly toxic. We have previously discovered the interaction of cancer cell surface proteins (including, urokinase plasminogen activator receptor (uPAR) and integrin αvβ6, shown as uPAR•αvβ6) that appears to have a crucial role/s in driving CRC metastasis. Here, we rationally designed and generated six interference peptides (iPEPs) as potential uPAR•αvβ6 antagonists. These iPEPs were tested on a CRC cell model and it was found that two (iPEPs 2/6) inhibited the growth and invasion, altered their morphology and switched key signalling events.

Methods

CRC cells (SW480β6OE, αvβ6 overexpressing) were treated with each of six biotinylated iPEPs or scrambled peptides. iPEP binding was monitored using a streptavidin-FITC conjugate and cytoskeleton actin changes was determined using phalloidin staining. Matrigel invasion and quantitative cellular proteome changes were performed on SW480β6OE and control SW480 cells with/without iPEP treatment.

Results

Fluorescence microscopy confirmed only iPEPs 2 & 6 bound to SW480 cells and increased actin "spicule" formation. Invasion assay upon iPEPs 2/6 treatment showed significant reduction in cellular invasion compared to non-treated controls. Total of 1,358 common proteins were identified across triplicate MS-runs between SW480β6OE cells with/without iPEPs treatment, of which 37 were downregulated and 67 upregulated ($p<0.05$, fold change >2). Ingenuity pathway analysis showed 88 cancer related proteins in this up/down regulated list and were enriched for morphology and cancer signalling pathways. Signalling switching to favour MAPK-dominant over SMAD pathways occurred after uPAR•αvβ6 expression.

Conclusions

Upon expression, uPAR•αvβ6 interactome drives CRC epithelial cells metastasis. iPEPs antagonising uPAR•αvβ6 disrupts processes associated with proliferation, invasion and growth, suggesting that iPEPs may conceivably represent a novel therapeutic strategy to curb CRC metastasis.

Keywords

Colorectal cancer, interference peptides, proteomics

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Insidious or ingenious: complexities of antigen presentation

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Background:
The cellular immune response relies upon T cell recognition of peptides presented on the cell surface in complex with HLA molecules (pHLA). The peptide cargo of HLA molecules dictates the quality of the immune response and ultimately the efficacy of protective immunity. Conversely aberrant antigen (Ag) presentation can lead to autoimmunity or allergy. The complexity of the Ag and the highly varied chemical entities that may form T cell ligands is only just beginning to be appreciated. This includes T cell recognition of post-transcriptionally and post-translationally modified peptides. We have used a comprehensive peptidomics approach to map antigenic complexity at the level of antigen processing and presentation.
Methods:
Our overlying motivation is that the combination of epitope discovery, structural immunology and bioinformatics capabilities with innovations in understanding Ag complexity will transform how we approach vaccination and immunotherapy across a range of infections, cancer and immune disorders.
Results and Conclusions:
In this presentation I will focus on the proteolytic processing of antigenic precursors that give rise to significant complexity in the HLA-class I (HLA-I) peptide repertoire (immunopeptidome) and how it impacts epitope trimming and epitope splicing both within the constitutive immunopeptidome and also during infection with pathogens such as HIV (1) and influenza.
Keywords:
Immunopeptidome, infection, post-translational modification, antigen presentation

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Phenotyping of rheumatoid arthritis patients by citrulline specific immunome protein arrays

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Background: Rheumatoid Arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the population worldwide. RA is characterized by inflammation of the synovial tissue, resulting in loss of function and joint degradation, which have severe consequences such as decreased mobility and increased morbidity. The autoimmunity is often associated with presence of anti-citrullinated protein antibodies (ACPAs), which serve as a diagnostic marker in RA. ACPAs bind to citrullination derived autoantigens resulting in local and systemic inflammation. Currently, there is no treatment for RA, however, a consensus of diagnosing RA as early as possible seem to be agreed upon. Therefore, we investigated targets of ACPAs in order to elucidate potential biomarkers.

Methods: Plasma samples from 15 anti-cyclic citrullinated peptides (CCP) positive RA patients, 10 anti-CCP negative RA patients, and 20 healthy controls were collected. The samples within each group were pooled and added to an immunome protein array containing 1631 different native folded proteins. The protein arrays were treated with and without PAD2 and PAD4 (Cayman chemicals) to citrullinate the proteins. Spot intensities were visualized using Cy3 anti-human IgG and measured using a microarray laser scanner (Innoscan 710). Data analysis was performed in Spotxel (SICASYS) and R.

Results: Preliminary results using PAD2 and PAD4 treated arrays on anti-CCP positive RA plasma samples identify 2 and 9 autoantigens against their citrullinated target, respectively. An overlap between already known autoantibodies and our results is present; however, we also identify several novel citrullinated autoantibody targets.

Conclusions: We demonstrate a functional setup that are able to identify immune-related targets that ACPAs bind. We identify already known targets, but also provide a list of novel autoantibodies present in RA patients.

Keywords: Citrullination – Rheumatoid Arthritis – Plasma – Protein Array

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μZIC-HILIC-MS/MS methodology for the characterization of N-glycan isomers.

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Background

Modifications in protein glycans have been related to many diseases such as cancer. Hence, characterization of N-glycan structures and their isobaric isomers is nowadays of great interest. With this aim, some studies have proposed the use of specific MS/MS ion fragments. However, most of reported methodologies are not able to separate glycan isomers prior to MS and reliably determine diagnostic ion fragments. The assignation of human alpha acid glycoprotein (hAGP) glycan isomers performed in our previous study, using exoglycosidase digestions in combination with relative quantitation by isotope labelling with [12C6]/[13C6] aniline, was used in this work to establish a reliable MS/MS method able to characterize glycan isomers.

Methods

hAGP purified from serum samples was digested with PNGase F and in some cases with total or α2-3 sialidase. Afterwards, N-glycans were purified, labelled with aniline and finally analyzed by ZIC-HILIC-MS/MS.

Results

The information of hAGP glycan isomers obtained previously with exoglycosidase digestions in combination with the ZIC-HILIC approach to separate glycan isomers and the exact mass of daughter ions provided by the LTQ-Orbitrap-MS, were used to confirm or discard the diagnostic character of several MS/MS ion fragments reported in the literature. hAGP glycans were deeply characterized and sialic acid / fucose linkage-type isomers were assigned with the selected diagnostic fragments. Finally, control and pathological serum samples were analysed to demonstrate the robustness of this MS/MS methodology.

Conclusions

Reliable diagnostic ion fragments were selected in this work for sialic acid and fucose linkage-type assignations. Moreover, the established method enabled the location of α2-3/6 sialic acids on antennas and a complete characterization of complex type N-glycans, which were only partially characterized with exoglycosidase digestions. Serum sample analysis demonstrated its potential to find novel glycan-based biomarkers in cancer research.

Keywords

Glycan / Isomer / Tandem mass spectrometry / ZIC-HILIC

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Comparing the CSF proteome of neuromyelitis optica patients to multiple sclerosis patients and control subjects

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Background

Neuromyelitis optica spectrum disorder (NMOSD) is a rare and heterogeneous disease of the central nervous system characterized by severe inflammation and demyelination. Demyelination observed in NMOSD differs from the demyelination in the related disease multiple sclerosis (MS), despite the clinical overlap between these patients. It is necessary to gain more insight in the different mechanisms of pathophysiology in both groups.

Methods

CSF of 28 NMOSD patients, 47 clinically isolated syndrome patients who were diagnosed with clinically definite MS during follow up and 112 control subjects was enzymatically digested and analyzed by high resolution mass spectrometry (Orbitrap QX+) after separation using a 90 minute LC gradient on a C18 column. The resulting spectra were analyzed using specialized software (Progenesis LC-MS) and the identified proteins were analyzed with stringent criteria to detect the significantly differentially abundant proteins between the groups. Subsequently the differentially abundant proteins were submitted to Ingenuity Pathway Analysis (IPA) for assessment of their connection and roles in biological pathways that might be relevant to disease pathology.

Results

In total we identified 6927 peptides belonging to 774 proteins, with a peptide FDR of 0.02% and a protein FDR of 0.4%. the comparison of NMOSD versus control subjects, 46 proteins passed the criteria for statistical significance between these two groups. Interestingly, all these proteins, which included three members of the Cathepsin family, were lower abundant in NMOSD than in control subjects. An additional 22 proteins, mostly related to the immune response, were significantly lower abundant in NMOSD compared to MS, indicating differences in the extent of inflammation between the two groups at the time of sampling.

Conclusions

These results indicate a different level or type of inflammation activity in NMOSD compared to MS, which may be of interest in the context of understanding the pathophysiology of both entities.

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Site-specific N-glycosylation analysis of human IgA isotypes produced in glyco-engineered plants by LC-ESI-MS

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Background:

Immunoglobulin A (IgA) is increasingly gaining attention as a biopharmaceutical for the treatment of infectious diseases and cancer. However, the full potential of recombinant IgAs as therapeutic antibodies is not completely explored, owing to the fact that structure-function relationships of different glycans attached to these extensively glycosylated proteins are not well understood.

Methods:
Various IgA isotypes were recombinantly produced in the glyco-engineered N. benthamiana ΔXT/FT line that essentially lacks plant specific β(1,2)-xylose and core α(1,3)-fucose residues. Affinity chromatography was used for purifying the clarified leaf extracts. After proteolytic digestion the samples were subjected to LC-ESI-MS to elucidate the N-glycosylation pattern of each site (2-5 depending on the isotype). In addition, the proline-rich hinge region of isotype IgA1 harboring up to nine potential O-glycosylation sites was examined as well.

Results:
All predicted N-glycosylation sites were found to be occupied in all three IgA variants investigated. N-glycans found on plant-produced IgA showed a comparably homogenous profile, with biantennary complex-type structures like GnM/MGn and GnGn (proglycan nomenclature, www.proglycan.com) as major glycoforms lacking the plant specific β(1,2)-xylose and core α(1,3)-fucose residues as expected. On the hinge region of plant produced recombinant IgA1 the conversion of proline residues to hydroxyproline and the presence of additional pentoses (presumably representing attached arabinose chains) were detected.

Conclusion:
All three IgA variants were expressed in the glyco-engineered Nicotiana benthamiana plant-based system that is, for example, used to manufacture the ZMapp antibody cocktail against Ebola virus infections[1]. The recombinant IgA subtypes were purified, biochemically and biophysically characterized and subjected to comprehensive site-specific glycosylation analysis to reveal common features as well as differences that may have implications for their function.

[1] Qiu X, Audet J, Lv M, He S, Wong G, Wei H, et al. Two-mAb cocktail protects macaques against the Makona variant of Ebola virus. Science translational medicine 2016

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Profound aging-related changes of histone H3 tails in mouse tissues

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Chromatin structure and function is maintained by dynamic protein-protein and protein-nucleic acid interactions. Histones are abundant chromatin constituents carrying numerous post-translational modifications (PTMs). Such PTMs mediate a variety of biological functions such as gene regulation, and are assumed to control the maintenance of specific cell types. Individual histone molecules contain multiple co-existing PTMs some of which exhibit crosstalk, i.e. coordinated or mutually exclusive activities that we recently showed to be conserved within different cell lines.
The study investigates histone PTM patterns and concurrent changes of chromatin modifiers in mouse tissues at different ages, in order to find global changes in chromatin induced by aging and to unravel the control mechanisms that distinguish and maintain cells in different tissues.
Histone H3 PTMs and their crosstalk were measured using a recently established middle-down mass spectrometry and bioinformatics pipeline allowing the quantification of multiple PTMs and their crosstalk. Mouse tissue (brain, heart, liver and kidney) samples at different ages were quantified and assessed by computational and statistical methods showing reproducible patterns of their histone PTM landscape.
Histone variant H3.3 exhibited dramatic increase at higher age where it substituted canonical histone variants H3.1 and H3.2. This exchange is associated with global changes within the PTM landscape including multiple PTMs known to regulate gene transcription. Moreover, expression profiles of chromatin-modifying proteins showed patterns well in line with changes of the histone PTM landscape.
Integrated experimental and computational analysis allows an in-depth view of the characteristic patterns in chromatin organization that define different ages and tissues. Particularly, histone variant H3.3 plays a crucial role in ageing cells suggesting a crucial role in modulating the histone methylation landscape throughout the lifespan.
All histone H3 PTM data will be available in our public CrossTalkDB repository at http://crosstalkdb.bmb.sdu.dk

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Structural proteomics uncovers the dymanic of DNA response element-Transcription factor interaction.

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Transcription factors (TF) regulate gene expression through interactions with DNA response element (DRE) in promotor region and other regulatory proteins. Our understanding of this regulation depends on the knowledge of the TF-DRE complex structure, which is accessible by the conventional methods (X-ray, NMR), yet the process is still very challenging. Thus, the development of faster alternative approaches allowing detailed description of a TF-DRE complex is beneficial. To design a reliable mass spectrometry technology, a palette of MS based approaches, including H/D exchange, protein-protein and protein-DNA cross-linking was applied to investigate the TF-DRE complex structure in solution.
DNA binding domain of TF was expressed in E. coli and purified utilizing affinity and size exclusion chromatography. The DRE representing oligonucleotide was synthesized. H/D exchange (HXMS) was followed for TF alone or in mixture with the oligonucleotide. Chemical cross-linking (CXMS) included both quantitative protein-protein (amino reactive reagents DSS and DSG in non- and deuterated form) and protein-DNA cross-linking (trans-Platinum(II)diamine dichloride). The analysis of HXMS and CXMS samples were performed by reverse-phase chromatography coupled to FT-ICR mass spectrometer (solariX XR 15T). HXMS and CXMS data were processed using own proprietary software DeutEx and LinX, respectively.
H/D exchange results were in agreement with the known high resolution structures of TFs obtained by NMR or X-ray. Moreover, significant changes (stabilization of the helix H2) upon DRE binding support the molecular dynamic simulations.Protein-protein chemical cross- linking revealed structural differences in several regions, especially in the unstructured part where the data span further beyond the known high-resolution structures. Further, several protein-DNA cross-links were identified explaining structural changes induced by ligand binding in intrinsically disordered regions.

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HD Proteome Base: A Novel Data Repository for Proteomics of a Huntington's Disease Mouse Model

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Background

Huntington's disease (HD) is a hereditary, neurodegenerative disease caused by an abnormal expansion of a glutamine stretch (polyQ) in the sequence of Huntingtin protein (HTT). Extensive research efforts are aimed at understanding molecular mechanisms altered in the presence of the aberrant HTT. To this end, we compared the proteomes between wild-type and heterozygous Huntingtin knock-in mice with increasing CAG repeat lengths in a number of different brain regions and peripheral tissues at three different ages.

Methods

A large-scale proteomic analysis was conducted on various brain regions (striatum, cortex, cerebellum, and hippocampus) and peripheral tissues (liver, muscle, and heart) from wild-type and heterozygous Huntingtin knock-in mice with increasing CAG repeat lengths aged 2, 6, and 10 months. For storage and query of results, a user-friendly web interface was created

Results
The analysis of more than 1,200 tissue samples with on average 8,000 quantified proteins comprises one of the largest global, quantitative proteomics studies published so far. More importantly, it allows a systematic analysis of pathways and interaction networks on the protein level, the identification of novel target candidates, and provides a comprehensive resource for training of system biology models. Here, we describe a novel data repository termed HD Proteome Base, which stores and displays the quantitative proteome profiles collected within this large-scale study. The repository will be publicly accessible through a web portal that allows the researcher to query for proteins and to visualize their expression across the CAG repeat length series and across different brain tissues as well as peripheral tissues.

Conclusions

Our novel, publicly available HD Proteome Base affords researchers around the globe to access and employ this very comprehensive resource and, thereby, contribute to research efforts towards gaining a better understanding of huntingtin biology.

Keywords: Huntington's Disease, Proteomics Data Repository, Huntingtin, Systems Biology, Data Resource

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Methylglyoxal-modified peroxiredoxin 6 as a biomarker for diabetic complications

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Methylglyoxal (MG) is an endogenous highly reactive dicarbonyl degradation product formed from triose phosphates during glycolysis, and forms stable adducts primarily with arginine (Arg), lysine (Lys) and cysteine (Cys) residues of proteins. An antioxidant protein peroxiredoxin 6 (Prx6) was found as the major MG-adducted protein in red blood cells (RBCs) from diabetic patients. Peroxidase activity of recombinant Prx6 was inhibited by MG modification in a concentration-dependent manner. Mass spectrometry analysis of peptide fragments of MG-modified recombinant Prx6 identified residues Arg-24, Arg-41, Arg-132, Arg-106, Arg-219, and Lys-63 as the modification site in the protein. Arg-132 is situated in the catalytic center of peroxidase activity. Prx6 contains one conserved reactive residue Cys-47 in its active site. The decrease in the number of free thiols in Cys residue upon incubation with MG was observed. These results suggest that the decrease in peroxidase activity is due to modification of active-site residues in Prx6. The level of MG modification on Prx6 was correlated to both fasting glucose concentration ($p<0.0001$) and postprandial glucose concentration ($p<0.0001$) in diabetic patients. This modification level was more sensitive than HbA1c against fasting glucose concentration ($p=0.0003$) and postprandial glucose concentration ($p=0.0001$). It was revealed that the level of MG modification on Prx6 does not change with time since diagnosis of diabetes. Moreover this modification level was higher in the hyperlipidomia patients than in the healthy control subjects. Therefore, it was suggested that protein modification by MG plays an important role in the development of diabetic complications.

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Absolute quantification of a membrane-localized chemosensory protein network

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Background

Determination of the precise stoichiometry of components for a signalling pathway network is key for insights of the signal relay mechanism in terms of quantitative systems biology. Whilst common quantification methods like AQUA, PSAQ and Qconcat possess advantages and disadvantages, recent reports suggest that Qconcat is probably best-suited for this endeavour. Here, we address the question if and how a sizeable membrane-localized protein network can be completely quantified at high fidelity with Qconcat. Our model system to test the virtues of Qconcat was a chemosensory pathway in sperm from the sea urchin A. punctulata. Our data provide quantitative insight into signaling and subunit stoichiometry of up to 19 different proteins.

Methods

Peptides suitable to generate a synthetic Qconcat protein were selected based on results of shotgun and targeted proteomics. The Qconcat peptide contained for each of the 19 proteins 2-4 proteotypic peptides for quantification. Sperm tail proteins and the Qconcat peptide were together subjected to SDS-PAGE and tryptic protein digestion. RP- LC separation and MRM analysis on a TSQ vantage, and Skyline software were used for quantification.

Results

In total, 94% of all theoretical Qconcat peptides could be retrieved in the analysis. Quantities differed between 2-4 used peptides for each protein between 2 and 50%, indicating the necessity of having at least two peptides to achieve accurate quantification. The protein abundances in the network covered a dynamic range of more than 200. The chemoreceptor guanylate cyclase was most abundant, and the Ca^{2+} channel (CatSper subunits) least abundant. In terms of precision and accuracy, our data demonstrate that PSAQ and Qconcat perform superior to AQUA peptides.

Conclusions

It is possible to achieve Qconcat performance statistics for a large network of membrane proteins similar to previously reported soluble proteins.

Keywords

network stoichiometry, membrane protein, Qconcat

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Label-free microarrays for optimisation of protein and antibody binding

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Background:
Precise knowledge of thermodynamic and kinetic constants like affinity and on/off rates of an antibody-antigen interaction is mandatory for many applications. Moreover, the extensive information about the specific sequence of the epitope the antibody binds to (epitope mapping) as well as the competition of different similar monoclonal antibodies for the specific epitope (epitope binning) provides important data for the discovery and development of new therapeutics, vaccines and diagnostic applications.

Methods:
Common epitope mapping and binning experiments are time and material consuming as they require a lot of subsequent measurements. A suitable high-throughput method for fast and cost-effective antibody characterisation would remarkably accelerate the antibody characterisation and development. SCORE technology (single colour reflectometry) is a predestined tool for such applications. It is a high-throughput label-free detection method that is compatible with standard microarrays. Up to 22,500 interactions can be analysed within one single measurement run within minutes.

Results:
Detailed results of an epitope mapping as well as an epitope binning study performed on the Biametrics b-screen device will be presented and the related advantages compared to common approaches. Epitope mapping was performed on variations of the FLAG epitope on a high-density peptide array. For the epitope binning study, a set of 7 antibody fabs and their spatial epitope arrangement has been investigated.
Also results of a label-free readout of high-density peptide microarrays using SCORE technology will be shown. The binding affinities of the protein Grb2 to more than 500 target peptides on the microarray were analysed.

Conclusion:
Its performance makes the b-screen the ideal tool for analysing high-density arrays of any kind of biomolecular interactions, ranging from peptides to (recombinant) proteins and antibodies up to whole cells. It is compatible with the standard microarray format and printing technologies.

Kewords: label-free, protein-protein interactions, kinetics, epitope binning, microarrays

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Targeted mass spectrometry to determine the stoichiometry of phosphorylation sites in proteins

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Background
There is a large interest in studying the phospho-proteome and changes that occur in the phospho-proteome in relation to various diseases. We describe a method to identify and quantify phospho-peptides and to determine their stoichiometry based on a combination of data dependent LC-MS measurement and a subsequent quantitative measurement using Parallel Reaction Monitoring (PRM).
Methods
U87 glioma cell line was used as a model. U87 cell pellets were collected and digested using trypsin. Next, phospho-peptide enrichment followed by high-pH reversed-phase fractionation was performed using AssayMAP Bravo platform followed by LC-MS analyses on a nanoLC Q Exactive HF. For PRM analyses 4 peptide pairs were selected (phospho-peptide and the non-phosphorylated counterpart). The selected peptides are: GSHQISLDNPDYQQDFFPK (ser 1166, EGFR), ESTESSNTTIEDEDVK (Thr 337, CAMK2D), SPPYTAFLGNLPYDVTEESIK (ser 93, EIF4B), ISAPNVDFNLEGPK (ser 5448, AHNAK) To validate our method, we performed immunoprecipitation for EGFR on the same amount of protein. Experiments were performed in triplicate.
Results
The resulting data files were analyzed using the Mascot search engine against the UniProt database and resulting in the identification of 2337 phosphoproteins and 7178 unique phosphopeptides (FDR 1%), with 6108 unique phosphosites. We developed a PRM method using stable isotope-labelled peptides to quantify the phosphorylation of the above-mentioned peptides. The phosphorylation ratios (EGFR) in digested U87 cells were measured by PRM, 4.4% (CV 7.1%) without affinity-enrichment compared to 4.9% (CV 14.3%) with affinity-enrichment for this EGFR peptide.
Conclusion
Using the untargeted-MS method, we are able to identify and quantify large numbers of phosphoproteins and phosphosites in a single sample and using the targeted-MS method, we could quantify the phosphorylation ratio of specific sites directly in crude lysates with high confidence.
Key words (5words): phospho-enrichment, fractionation, PRM, phosphorylation ratio, EGFR

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Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma

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Background: Cholangiocarcinoma (CCA) includes a heterogeneous group of biliary cancers with poor prognosis. Several conditions such as primary sclerosing cholangitis (PSC) are risk factors. Accurate non-invasive biomarkers for CCA or PSC are not yet available. Lately, extracellular vesicles (EV) have attracted increasing attention in the search of biomarkers for different disorders as well as their pathogenic role involved in disease development and progression.

Methods: Serum EV were isolated from CCA (n=13) or PSC (n=9) patients and healthy individuals (n=10) using well established ultracentrifugation/filtration methods. In addition, EV were isolated from the culture medium of normal human cholangiocytes (NHC), SV-40 immortalized human cholangiocytes (H69) and two CCA human cell lines (i.e. EGI1 and TFK1). The characterization of EV was performed by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and immunoblot. EV proteomes were digested by FASP method and analyzed by label free nLC MS/MS on nano Acquity coupled on-line to Orbitrap /Synapt G2Si.

Results: Round morphology (by TEM), size (~165 nm diameter by NTA) and bonafide EV markers (CD9, CD63 and CD81 by immunoblot) indicated that most serum EV were exosomes. Proteome profiles (by mass spectrometry) revealed 128, 121 and 43 proteins differentially expressed in CCA vs control, PSC vs control, and CCA vs PSC groups, respectively. Among them, several proteins showed high diagnostic values in terms of sensitivity, specificity. The proteomic analysis of EV isolated from CCA human cells in vitro revealed higher abundance of oncogenic proteins compared to EV released by NHC.

Conclusions: Novel proteomic signatures found in serum EV of CCA and PSC patients show potential usefulness as diagnostic and prognostic tools. CCA-derived EVs contain increased concentration of oncogenic proteins that might participate in tumor progression. Further validation experiments with larger sample cohorts are under study.

Keywords: extracellular vesicles, cholangiocarcinoma, primary sclerosing cholangitis, biomarker, diagnosis.

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Extracting Peptide Level Quantification data from the PRIDE Repository

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Background
PRIDE is the world's leading repository for mass spectrometry based proteomics data, and hosts the acquired mass spectra and corresponding peptide and protein identifications in a queryable form for thousands of submitted experiments (Vizcaíno et al. 2016).
However, peptide ion intensity based (MS1) quantification results are not available in PRIDE for querying, and even if available (in files) can be difficult to compare across data sets due to the heterogeneity in quantification tools and analysis protocols used. We therefore developed moFF (Argentini et al. 2016), a robust apex-based tool to extract MS1 intensities that has equivalent performance with MaxQuant(Cox & Mann 2008), yet is highly automatable and does not suffer from operating system limitations. moFF can therefore be used in a pipeline to extract uniform and comparable MS1 intensities from PRIDE data.
Methods
A pipeline is built based on moFF to provide MS1 intensities for the identified peptides as provided in "complete" submissions in PRIDE. The process is structured in three steps: (1) extract retention times for all spectra from the raw files; (2) quantify the precursor peptide for each spectrum using moFF (3); associate the extracted MS1 intensities to the identified peptides. The pipeline runs on a Linux cluster environment and is fully parallelizable.
Results
All MS1 intensities collected across the PRIDE experiments will populate a dedicated online database. This will be a very useful resource for researchers in the field, allowing applications such as the detailed investigation of peptide detectability, or the use of quantitative peptide data in genome annotation pipelines to aid in assessing the most likely protein isoform.
Conclusions
The proposed moFF-based PRIDE reprocessing pipeline will be used to create a valuable resource for the whole community, with exciting possibilities for downstream use.
Keywords
peptide-quantification, re-processing, bioinformatics

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Gut-Brain Axis in Ageing: A Proteomics Approach

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Background. Increasing evidence has suggested that alterations to the epithelial barrier of the gastrointestinal (GI)-tract are linked to both local and systemic chronic disorders affecting various organ systems including the brain. The small intestine changes during the life course in healthy young, adult, and ageing individuals1. Accumulating evidence suggests that ageing is associated with a chronic low-grade inflammatory2 that may affect brain function too. AIM. This project is focused on the hypothesis that age-related changes in the intestinal microbiota directly contribute to age-associated alteration of the intestinal barrier integrity, brain morphology.
Methods. C57BL/6 strain mice underwent faecal microbial transplant (FMT): 1) In the Causation experiment adult mice underwent aged faeces transplant to study the ageing induction of aged microbiota on adult mice in the gut and in the brain. 2) In the Therapy experiment aged mice underwent adult faeces transplant to study the potentially ageing regression in the gut and in the brain. The control aged mice underwent aged faeces transplant and no transplant at all. The FMT were performed in both experiments by 5 oral gavage feeding and the mice were sacrificed after 12 days. The collected organs were subjected to label free quantitative Proteomics approach.
Results / Conclusions. In the brain the pathways involved during the induction of ageing process (Causation experiment) were related to "Ras signalling pathway", "Inflammatory mediator regulation" and Endocytosis. In the brain, after Therapy experiments, Calmodulin, Arp2/3, and protein phosphatase 2A, were found up regulated related to controls. In conclusion this is the first protein profiling descriptions of gut and brain after FMT and related to ageing processes.
Keywords: Gut-Brain Axis, label free, protein networks
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Individualised Proteogenomics in Analysis of Single Amino Acid Variants in Cancer

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Background: One cancer type often results from an accumulation of different mutations between affected individuals, which calls for personalised approaches to cancer understanding and treatment. These somatic mutations can lead to altered protein sequences, which in turn result in disturbed signal transduction networks. In melanoma, ~66% of cases carry somatic mutation V600E in BRAF kinase, which leads to constitutive activation of MAP kinase signalling pathways. Recently, kinase inhibitors (vemurafenib) showed great promise in melanoma treatment, however, treated tumours inevitably develop resistance. Although mechanisms of resistance have been proposed, key proteins and associated mutations responsible for different therapy responses are largely elusive.
Methods: We have established a bioinformatics workflow to predict nsSNVs, InDels and frameshifts from exome sequencing data of analysed samples, and incorporate these mutations into individualised protein sequence databases. This workflow was applied to investigate vemurafenib-sensitive and -resistant A375 melanoma cells at the exome, proteome and phosphoproteome levels in order to dissect the influence of somatic mutations on resistance development.
Results: Using sequencing data from drug-resistant and -sensitive A375 melanoma cells, we detected 20,554 missense mutations, of which 5,686 were not previously reported. Mutations were classified based on their potential effect: location on modifiable amino acid (Ser/Thr/Tyr/Lys), location within known motifs, location on known modification sites and assignment to recognised cancer-relevant protein. A scoring system was established to rank mutations based on their predicted impact. Among highly scored mutated proteins were YES1, regulating cell growth and survival; and ABCB10, involved in drug resistance and cell proliferation. We are currently validating several mutations by sequencing and MS to confirm their involvement in vemurafenib treatment resistance.
Conclusions: Our proteogenomics workflow provide insights into molecular mechanisms underlying resistance to BRAF inhibitors and can be applied to personalised approach to cancer characterisation in cell lines and patient tissues.
Keywords: Melanoma, Cancer, Proteogenomics, Mutations.

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Synthetic Peptide Reference Standards for PTM Proteomics

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Background
Although mass-spectrometry based proteomics has emerged as a powerful method for the analysis of post-transitional modifications (PTMs), PTM proteomics is still challenging.[1] Typical problems associated with PTM peptides are a) low endogenous abundance, b) low ionization intensity, c) strong fragmentation, d) limited stability during proteomics workflows, and/or e) complex fragmentation spectra interpretation, especially regarding correct PTM site localization.
Irrespective of the described issues defined synthetic PTM reference peptides represent valuable tools to support increasingly important PTM proteomics and help to overcome challenges. Accordingly, the recently launched ProteomeTools project[2] will incorporate more than 300.000 PTM modified peptides.

Methods
Various sets of PTM peptides were synthesized and analyzed by LC-MS. The PTMs were incorporated by using respective pre-synthesized modified amino acid building blocks. A subset of the synthesized peptides was prepared in a stable-isotope labeled (SIL) form, purified to high purity and absolutely quantified.

Results
>10,000 Peptides with the following frequently analyzed PTMs were successfully synthesized and analyzed by LCMS: Phospho-Ser/Thr/Tyr, Lys(Acetyl), Lys(GG) as a surrogate for ubiquitinylated Lys.
In addition, a number of modified peptides with less frequently analyzed or even only recently discovered PTMs were prepared: Arg(Me), Arg(Me2), Lys(Me), Lys(Me2), Lys(Me3), Lys(Formyl), Lys(Propionyl), Lys(Butyryl), Lys(Succinyl), Lys(Malonyl), Lys(Glutaryl), Lys(Crotonyl), Lys(Hydroxyisobutyryl), Lys(Biotinyl), Sulfo-Tyrosine.
Finally, also a number of glycosylated peptides were prepared: Ser/Thr(alphaDGalNAc), Asn(betaDGlcNAc).

Conclusions
Sets of PTM modified peptides were prepared to support PTM proteomics with reference standards. These should be valuable tools for optimizing and standardizing proteomics workflows and analysis conditions.

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An integrative multi-omics approach for improved discovery of biomarkers related to prostate cancer progression.

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Background
Prostate cancer is the second most common cause of cancer-related death in the UK, which is primarily due to metastasis. A process known as epithelial-mesenchymal transition (EMT) is highly implicated in the spread of cancer. This involves epithelial cells of the primary tumour changing to a mesenchymal cell type. These cells detach and disperse via the bloodstream into distant sites in which they initiate the growth of secondary tumours. Here, we present the use of two inducible EMT models, which have been used to improve our understanding of metastasis in prostate cancer and the discovery of potential biomarkers for disease progression.

Methods
The highly epithelial clone P5B3 (derived from OPCT-1, a prostate cancer cell line generated from a primary tumour) and the metastatic prostate cancer cell line DU145 were stimulated with TGF-β, which induced EMT. Total RNA and proteins from 10 biological replicates of control and treated cells were analysed using a SCIEX TripleTOF™-6600 mass spectrometer and quantitative SWATH-MS™ profiling or an Illumina NextSeq500 sequencing platform. Resulting gene/protein lists were significance filtered and the remaining candidates were applied to artificial neural network (ANN) based network analysis.

Results
This approach highlighted 235 genes and 18 proteins, which were present in both models. 13 potential markers were found at both the transcriptomic and proteomic level, which suggests a high association of these markers with EMT in these inducible models. Preliminary data demonstrating expression of these candidate markers in a normal tissue RNA panel, suggests that some are expressed at low/negligible levels.

Conclusions
This combination of methodologies has supported the identification of potential genes and proteins involved in EMT independently from published information. The future work will focus on the verification and validation of the selected candidates using clinical material to identify their diagnostic/prognostic value.

Keywords
Integration, multi-omics, prostate cancer, metastasis, EMT

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Identification, validation and characterisation of membrane protein tumour markers for pancreatic adenocarcinoma

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Background
Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer related deaths worldwide and develops in a relatively symptom-free manner leading to rapid disease progression and usually a dismal prognosis at the time of diagnosis. Targeted therapeutics has dawned a new era of cancer treatments such as the introduction of antibody-drug conjugates (ADCs) which take advantage of the specificity of antibodies to deliver cytotoxic agents to cancer cells expressing a tumour specific protein marker. This project is focused on the identification of membrane proteins associated with PDAC for their use as novel targets for ADC therapy.
Methods
Tissue homogenisation followed by membrane protein enrichment was performed on 20 primary pancreatic tissues (malignant n=10 and normal-adjacent n=10), 20 patient-derived xenograft samples from SCID mice (F1 generation n=10 and F2 generation n=10). Label-free LC-MS/MS analysis was performed on a nano-HPLC coupled to an LTQ Orbitrap XL using a 180-minute gradient. Quantitative data analysis was carried out using Progenesis QI for Proteomics whereby a protein was considered differentially expressed if it is statistically significant $p \leq 0.05$, has ≥2 peptides contributing to the identification and has a minimum fold-change between samples groups of 1.5-fold.
Results
335 proteins were found to be differentially expressed (DE) between the tumour samples and normal-adjacent, of which 166 had the highest abundance in the tumour samples. Using species-specific peptides for protein identification in the PDX analyses, over 150 human proteins were DE between tumour and F1 generation and 24 proteins DE between F1 and F2 generations.
Conclusions
This analysis presents strong target proteins identified from primary tissues of PDAC which have potential use as therapeutic targets or as predictive, diagnostic or prognostic biomarkers. Proteomic profiling of PDX models also highlights their relevance and importance in studying this rare carcinoma.
Keywords
Pancreatic adenocarcinoma; membrane proteomics; antibody-drug conjugates; patient-derived xenografts

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Applying proteomics to build a precise, predictive test for diabetic kidney disease

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Background

Diabetes is becoming the greatest health challenge of the 21st century. According to the International Diabetes Federation there were 415 million people worldwide with diabetes in 2015. Chronic kidney disease is a significant complication of diabetes that affects one in three patients, leading to dialysis or kidney transplant, and in 15% of diabetics, death from kidney failure. Current measures of kidney disease (measurement of albuminuria and/or estimated glomerular filtration rate (eGFR)) cannot predict future decline in kidney function. Clinicians require better tools to deliver more precise diagnosis and monitor treatment to improve health outcomes.

Methods

The role of previously identified diagnostic protein biomarkers (APOA4, APOC3, CD5L, C1QB, CFHR2, IBP3) was investigated in predicting kidney function decline in type 2 diabetes. Targeted mass spectrometry was used to measure plasma biomarkers at entry in 345 participants from the longitudinal observational Fremantle Diabetes Study in Western Australia. Kidney function was followed over four years. Multiple logistic regression identified clinical predictors of developing disease. The incremental predictive value of protein biomarkers was then assessed to determine the most parsimonious model. The resulting test, termed PromarkerD, was validated in an independent cohort (n=447), also over four years.

Results

The simple endpoint from the study showed a novel protein biomarker panel could correctly predict 95% of otherwise healthy diabetics who went on to develop chronic kidney disease within four years. A primary clinical endpoint from the study determined a ≥30% fall in eGFR over four years, and the statistical results showed that PromarkerD had Sensitivity 87%, Specificity 79%, and AUC 0.88.

Conclusions

The present study has used targeted proteomics to identify novel plasma biomarkers (APOA4, IBP3, CD5L, C1QB) that improve prediction of chronic kidney disease in diabetic patients independently of conventional clinical variables.

Keyword

diabetes targeted biomarker kidney disease

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Cross-linked peptide identification: a single answer from many tools?

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Background
Nowadays, mass spectrometry-based cross-linking (MS-XL) has become an important actor in unravelling protein structure, dynamics and complex formation. Correspondingly, many computational tools are available to identify cross-linked peptides as many groups have built their own tool, often tailored for a specific cross-linking strategy, or even for the specific cross-linker used within that group. Despite the many different tools in use, little is currently known about the overlap of the results obtained by these different tools. We therefore performed a thorough comparison of available tools, to study their overlap and/or potential biases.

Methods
A publicly available cross-linking dataset from ProteomeXchange (PXD002142) was used as the basis for comparison. This dataset originates from a cross-linking experiment on human serum albumin with BSR as cross-linker, followed by a tryptic digest, and measured on an LTQ-Orbitrap.

Results
Cross-linked peptides were identified from the measured spectra with ECL, StavroX, pLink, MassAI, kojak and Xilmass. The cross-linked peptides identified by each tool were subsequently compared with those identified by each other tool. This comparison teaches us that no two tools could identify the same set of cross-linked peptides. Moreover, the overlap in identified cross-linked peptides can even be very small for some of the tools, indicative of strong built-in biases.

Conclusions
Our result show that currently available tools are not able to provide a single, unified set of identified cross-linked peptides. The difference between the algorithms is most likely explained by the observation that most tools are very specific towards a cross-linker or cross-linking strategy. Moreover, it seems that instead of relying on only a single tool, the use of a combination of different identification algorithms is likely the best approach to obtain all possible information from a cross-linking experiment.

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Plasma proteome analysis enables accurate quantification of liver-secreted proteins

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Background
The liver plays a key role in overall metabolism and physiology. Malfunction of the liver may result in any of over 60 liver diseases and conditions. Chronic liver disease is usually asymptomatic and can progress unnoticed to cirrhosis and even hepatocellular carcinoma.
Proteins released/secreted from the liver may serve as biomarkers reflecting the state of the liver. Here, we describe a mass spectrometry-based plasma proteome profiling workflow that enables accurate quantification of liver-secreted proteins.
Methods
Plasma samples were prepared by an automated plasma proteome profiling pipeline and analyzed on 45-min HPLC gradients. The methods were built on our previously established Plasma Proteomic Profiling workflow (Geyer et al.PMID27135364). Coefficients of variation (CV) of LFQ values were calculated for individual proteins among technical and biological replicates.
Results
In total, we quantified 1,400 plasma proteins of which 121 were classified as “elevated in the liver”, which means that their mRNA expression level according to the Human Proteome Atlas was 5-fold higher in the liver than the average level of all other human tissues. These elevated in the liver proteins span over 5 orders of magnitude in abundance and were reproducibly quantified. We further quantified 76 proteins classified as “liver-enriched proteins”, which have an even higher liver specificity. They include markers associated with fatty liver disease and fibrosis such as alpha-2-macroglobulin, apolipoprotein A1, laminin, collagen VI, etc. In label-free quantitation and triplicate analysis overall CVs of these two categories of proteins are 25.3% and 22.5%, respectively. 65.8% of liver-enriched proteins achieve a CV of less than 20%.
Conclusions
This workflow is a first step towards the unbiased study of liver proteins in the circulation. It could be of great clinical interest and is already available for plasma biomarker discovery in the context of liver diseases.
Keywords
Plasma proteomics, mass spectrometry, liver disease, biomarker

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MHC peptidomics provides a new outlook on its production and presentation pipeline

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Background: The MHC peptidome is the assortment of peptides presented at the cells' surface and the significant interest in this peptidome stems from its potential usefulness as a source for cancer immunotherapeutics. The MHC peptidome is a product of the cellular proteins degradation and is sculptured by its processing pipeline and by the peptide binding specificities of the MHC allomorphs. The MHC molecules are the most polymorphic molecules in the human population, and therefore, each of the thousands of human MHC allomorphs (HLA) presents a vastly different repertoire of peptides.
Methods: We performed large-scale MHC peptidomics based on immunoaffinity purification of the MHC molecules and LC-MS-MS analysis of their bound peptidomes. We used different human and animal tissues, as well as cultured cells. We also used recombinant expression of different HLA alleles, as membrane anchored or as soluble molecules, with and without the presence of the enzyme and chaperones involved in their processing and assembly.
Results: The resulting data provides new insight on the molecular properties of the MHC-peptides bi-molecular interactions and on their processing and presentations processes. The collected data indicates that only part of the HLA class-I peptides are exposed to ERAP1 processing and points to the presence of differential limiting bottlenecks for the peptidomes presented by the different HLA alleles.
Conclusion: Large-scale MHC peptidomics is the only way that can lead to unbiased understanding of the molecular pathways that lead to the production and presentation of the unique MHC peptidomes
Keywords: Immunopeptidomics, antigen processing, HLA, MHC

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Proteomics of activated macrophages identifies novel innate immune signalling pathway promoting phenotypic switch in obesity

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Macrophages are phagocytic cells that show a high degree of diversity and plasticity that enable them to polarize in response to cues of the environment. These activation states also prepare macrophages for the uptake of exogenous microbial or endogenous apoptotic or necrotic cells. In many diseases such as diabetes, dysregulated metabolism induces chronic inflammation. So switch macrophages in obesity their phenotype from anti-inflammatory activation to a pro-inflammatory in the adipose tissue. However, the mechanism for this switch is unknown. In order to understand how activation affects macrophage and phagosome functions, we performed a comprehensive analysis of the total cell proteomes and phagosome proteomes from six activation states (resting, IL4, IL13, IL10, Interferon-γ, reprogrammed). Our data indicates that alternative activation by IL4, IL13 and IL10 change metabolic and phagosome functions substantially, leading amongst other things to enhanced lipolytic capabilities, thus preparing them for the uptake of large numbers of dead cells. Furthermore, our data shows that alternative activation by IL4 induces K63 polyubiquitylation of macrophage scavenger receptor (MSR1/SR-A) which recruits the TAK1/MKK7/JNK kinase signalling complex. Upon MSR1 ligation this leads to enhanced pro-inflammatory JNK activation thereby inducing a switch of the macrophage phenotype. We demonstrate that this MSR1 signalling is essential for switching macrophages from anti-inflammatory to a pro-inflammatory phenotype in the adipose tissue of obese mice, probably through ligation of MSR1 by apoptotic adipocytes. Our work established for the first time that MSR1 signals directly through JNK and provides a mechanism how macrophages switch phenotype in obesity leading to chronic inflammatory diseases such as diabetes.

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Proteomic toolbox for understanding the interaction between nanodrugs and biomolecules

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Background: The hidden language that leads the interactions between nanoparticles (NPs) and biomolecules and defines the NPs function has not yet explored deeply. Investigations have been directed either to synthesize NPs, or to evaluate responses to NPs exposure. Proteomics-based methods could elucidate the function of NPs in a more realistic scenario.

Methods: We have explored three proteomics based method to explore the interactions between NPs and biofluids and NPs and cells. We applied label-free shotgun proteomics to analyze the composition, distribution and dynamics of proteins adsorbed to NPs in contact with biomolecules. We have applied surface proteomics to identify the proteins at the surface of the protein corona that has entered the cell and elucidate their process through the vesicular pathway. We have applied quantitative proteomics to integrate the molecular and cellular response to NP uptake. Those studies has been performed with TiO2 and ultrasmall TiO2 –NPs, endothelial cells and endothelial cell media.

Results and discussion: Our results showed that TiO2-USNPs and TiO2-NPs along with its protein corona were internalized by the endothelial cells. Thereby several proteins from the corona escaped lysosomal degradation. We show that the NPs uptake modulate endothelial function by altering the endothelial permeability, cellular traffic and cell adhesion. TiO2-USNPs cause an orchestrated action on protein complexes involved in cell-cell adhesion, vesicular transport, and actin-cytoskeleton. The dissection of the molecular and cellular dynamics after NPs uptake could be applied to the development of nanomedicines with specific aims including modulating endothelial transcytosis, the strength of the endothelial junctions or the overall permeability of the endothelium. On the other hand the analysis of the evolution of TiO2-NPs protein corona in the transit from their initial interaction with biomolecules to the interaction with endothelial cells proteins showed the distribution and evolution of this corona proteins. Our data indicates that key factors to evaluate are microenvironmental physico-chemical properties and enzymatic processes in the route from administration-to target.
Conclusion: The basic research on the NP- biomolecule interface should be centered on the bio-physico-chemical factors that guide the evolution of the NP-corona in biological environments.

Keywords: shotgun proteomics, surface proteomics, nanoparticles

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A Leap over the Hurdle in Label-Free Quantitative Proteomics: Uniting Spectral Counting and Peak-Intensity-Based Methods

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Background

Data analysis strategies for label-free quantitative (LFQ) proteomics are typically based on either spectral counting (SC) or peak intensities. In SC, the number of peptide-to-spectrum matches for a given protein are counted. SC is intuitive and easy to apply but ignores a large part of the information available in high-precision mass spectra and does not accurately detect low fold changes. Contrary, peak intensity-based methods use maximum intensities or areas under the peak as proxies for peptide abundances and produce more precise protein abundance estimates. Goeminne et al. (2015) and (2016) showed that robust linear regression methods modeling log-transformed peptide intensities provide optimal sensitivity, specificity and fold change estimations. However, intensity-based methods cannot quantify proteins present in only one condition. Hence, key proteins that are largely affected by treatment go unnoticed.

Popular software routines partially overcome this problem by adopting imputation strategies under the "missing by low abundance" assumption, but this reduces the power to quantify other interesting proteins that are moderately to highly abundant (Goeminne et al., 2015).

Methods

We unite the advantages of SC and intensity-based peptide-level linear models in a novel hurdle model for LFQ proteomics consisting of two components: (1) a model assessing differential presence and (2) a robust regression model for differential abundance estimation given the observed peptide intensities for a protein.

Results

We compare our hurdle model to state-of-the-art methods for SC and intensity-based peptide-level analysis in a spike-in study and a real case study. Our method can quantify proteins that are only present in one condition, while providing an accurate, powerful assessment of differential abundance for proteins taking the presence hurdle in both conditions.

Conclusions

Our novel approach provides an optimal middle ground between SC and peak intensity-based methods enabling proteome scientists to uncover relevant proteins.

Keywords

LFQ, differential protein abundance, protein presence-absence

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Parallel accumulation – serial fragmentation (PASEF) on a trapped ion mobility spectrometry QTOF instrument

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Background: In data dependent acquisition experiments only around 20% of the eluting peptide features are targeted by current mass spectrometers due to limitations in sequencing speed, sensitivity and resolution. We recently described the parallel accumulation – serial fragmentation (PASEF) method that has shown promise to increase the sequencing speed and sensitivity of MS/MS scans on a prototype TIMS-QTOF instrument (Meier et al., JPR 2015, PMID: 26538118). Here we show further data acquired with PASEF-TIMS-MS in shotgun proteomics experiments.
Methods: In a TIMS-QTOF instrument ions are accumulated for a user-defined time and are released from the TIMS device (TIMS scan) dependent on their mobility cross section. By applying the PASEF method multiple precursors per TIMS scan were selected by sub-millisecond switching of the quadrupole isolation window. Raw data were analyzed using DataAnalysis (Bruker Daltonics) and MaxQuant (Cox group, MPI of Biochemistry).
Results: Different accumulation and release times (25 ms, 50 ms, 75 ms and 100 ms) corresponding to median ion mobility resolutions of 22 (25 ms) up to 78 (100 ms) were tested and sensitivity was improved by targeting low abundant precursor ions several times. To test if we can profit from the high speed and sensitivity of PASEF, we analyzed as little as 200 ng of a human cancer cell line (HeLa) protein digest on a prototype TIMS-QTOF mass spectrometer and a 90 min gradient. From this run we detected 200,000 peptide features and with only 12 ng of HeLa digest more than 50,000 peptide features were detected. Application of PASEF allowed the selection of 13 precursors within 100 ms release time, demonstrating the fast targeting of peptide features for MS/MS scans.
Conclusions: Our results demonstrate the use of PASEF-TIMS-MS for fast and sensitive acquisition in shotgun proteomic experiments and the potential for routine analysis of low sample amounts.

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Expression and Association of CDK10 with ETS2 proteins during Human Corneal Epithelial Wound Healing

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Background:
Integrity of the corneal epithelium depends the health of cornea which are maintained by different factors like corneal architecture, tear quantity, quality and corneal sensitivity. Altering any of these elements can lead to a corneal epithelial defect. Corneal wound repair and related complications are major health concern worldwide and could lead to loss of visual acuity. Therefore, there is an urgent need to develop reliable understanding of the underlying mechanism of corneal epithelial wound healing to apply therapeutic options. We aimed to investigate the alterations in protein expressions during corneal epithelial migration to demonstrate the networks of the total identified proteins with potential dual functions.
Methods: In this study, human corneal epithelial cells lines have been used for wound healing model. Mechanical wound was made in Cell lines and rate of healing was monitored at different hrs of post wounding using TScratch software tool. Epithelium was scrapped at each hours, followed by protein extraction and quantification. The proteins extracted from wounded and unwounded model at each hours were subjected to SDS-PAGE and 2-DE. Mass Spectrometry was done to identify the proteins through protein Matrix Science database searches. The identified proteins were further validated by western blot analysis. To further elucidate the potential biological function of identified proteins CDK10 and ETS2 proteins ectopically added in cell culture. Functional association involving the identified proteins in wound healing, was observed by IPA and STRING software.
Results: A significant finding of the present study is the identification of Cdk10 and ETS2, in healing corneal epithelium at active phase of migration. Interaction association network analysis further confirms the close interacting relationship between CDK10 and ETS2 proteins.
Conclusion: The present communication provides for the first time new evidence for the potential role of identified proteins in migrating epithelial cells as promoter .

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Decoding site-specific alteration of Sialo-glycoproteome in EGFR-subtype of non-small cell lung cancer towards precision medicine

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Background
Altered sialylation of cell surface glycoproteins has been correlated with EGFR mutation of non-small cell lung cancer (NSCLC) and tyrosine kinase inhibitors (TKI) resistance. Due to extreme heterogeneity, variable branching and extension of glycans, decoding the complex site-specific glycan structure on glycoproteins still presents great analytical challenges in mass spectrometry-based glycoproteomics. A highly specific enrichment and quantitation approach for intact glycopeptides is critical for comprehensive sialo-glycoproteomics profiling. Here, we report a new glycopeptide enrichment material, ZIC-cHILIC, and incorporating an isotopic labeling approach to decode the altered sialo-proteome in different EGFR subtypes of NSCLC cells.

Methods
The membrane proteins were extracted from different NSCLC cells. After in-solution digestion and labeled by TMT6plex, glycopeptides were enriched using ZIC-cHILIC stage-tip and eluted by stepwise fractionation. Intact glycopeptides were analyzed by HCDpd-stepped HCD using Orbitrap Fusion MS and identified by Byonic and quantified by PD2.1.

Results
Counting sialic acid-specific diagnostic oxonium ions in MS/MS spectra, cHILIC strategy showed as high as 88% specificity for enrichment of sialo-glycopeptides. On the sialo-glycoproteomic scale, 2346 unique intact sialo-glycopeptides from 747 proteins were quantified by TMT6plex labeling. The comparison of the 4 NSCLC cell lines with different EGFR subtypes revealed 29 unique glycopeptides site-specifically present in wild-type NSCLC, i.e. integrin-a3/b1 with fucosylated monosialo-biantennary glycan on N86/N363. Moreover, 19 and 24 glycopeptides were uniquely identified in NSCLC cells with exon 19 deletion and L858R/T790M mutation, respectively. The proteome-scale profiling also revealed high glyco-site identification coverage of individual glycoprotein. Without immunoprecipitation, 11 of 13 N-glycosylation sites on EGFR were identified and altered site-specific sialylated glycoforms were also quantified.

Conclusions
This quantitative sialo-glycoproteomic approach provides high specificity to map the global site-specific glycosylation profile and its changes. On the model study of NSCLC, our results may allow better understanding how the glycosylation correlates with EGFR subtypes.

Keywords
Sialylation, sialo-glycoproteome, NSCLC

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Extent of growth-related and drug-induced proteome changes in cancer cells

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Background
Mass spectrometry based proteomics is extensively used to evaluate the changes occurring in cellular proteomes upon drug treatment, for e.g. deconvolution of drug targets and mechanism of action. However, proteomes of cultured cells undergo also natural variation associated with changes in the media, attaining a degree of confluence as well as due to cell and circadian cycles. These changes are often explicitly assumed to be smaller in magnitude than the drug-induced changes that ultimately lead to cell demise. In this study, we aimed to test this assumption.

Methods
We test the above assumption by comparing the proteome dynamics of HCT116 colon cancer cell line under treatment with methotrexate, paclitaxel or vehicle (equivalent to untreated control). Samples were collected in duplicate every 6 hours for over 48 hours. Proteins were extracted and prepared following a bottom-up strategy (reduction/alkylation followed by LysC and tryptic digestion). Resulting peptides were directly injected in a Q-Exactive Plus mass spectrometer for MS/MS after separation on a LC system. Protein quantification was performed using MaxQuant software. The experiment was repeated with A375 malignant melanoma cell line in the absence of chemical treatment using 12 hours intervals.

Results
Time series analyses of abundances of 4700 proteins revealed considerable growth-related proteome changes in the control group, comparable in magnitude to drug-induced changes. A375 cell line showed similar variations on the same time-scale. We also observed that the targets of methotrexate and paclitaxel showed increased variations with time.

Conclusions
These observations highlight the importance of using proper control when measuring and interpreting the drug-induced proteome changes. We also show that long treatments are preferable for monitoring the drug effect on its target.

Keywords
FITExP; mass spectrometry; proteomics; principal component analysis; time series

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Imaging Mass Spectrometry of Kidney from Type 2 Diabetes (T2D) Rat Model

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Background A new inbred rat strain, Spontaneously Diabetic Torii (SDT) rats are excellent animal models to enhance our understanding of the diabetic nephropathy. Here we demonstrate a comprehensive matrix-assisted laser desorption/ionization (MALDI) mass imaging mass spectrometry (IMS) approach to study the molecular distribution of proteins in diabetic kidney using SDT.
Methods Kidneys from SDT rats at 5 and 10 weeks of age were resected and snap-frozen in liquid nitrogen. 10 µm cryosections were cut and transferred to Indium-Tin-Oxide (ITO) coated glass slides. Sinapinic acid was uniformly deposited on the slide using the ImagePrep device and measured using the ultrafleXtreme and rapiflex tissuetyper with a spatial resolution of 20 to 100 µm in linear mode. Ions were detected in a mass range of m/z 2000 to 20000. For Protein ID experiments, trypsin was sprayed at room temperature using the same apparatus. The multivariate analysis including probabilistic latent semantic analysis (pLSA) was done for obtained data.
Results In SDT rats, the relative kidney weights significantly increased after 14 weeks of age, as compared with those in age-matched SD rats. In this study, in order to find early proteomic markers of minimal diabetic renal insufficiency, we have analyzed SDT rats at 5 and 10 weeks of age by comparing with those from SD rats. Here we have shown the evidence of early changes of in situ renal proteome at 5 weeks of age when histopathological changes such as basement membrane thickening in the glomerulus are not prominent. Deeper analysis using supervised statistical evaluations revealed a number of candidate markers directly off-tissue that may be related to early diabetic nephrotic changes.
Conclusions Comprehensive proteomic IMS on kidney sections from SDT rats will clarify early diabetic nephrotic changes at peptide and protein level.
Keywords Imaging Mass Spectrometry, Type2 diabetes model, SDT rat, kidney

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Validation of antibodies using orthogonal methods

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There is a need for standardized validation methods for antibody specificity and selectivity for Western blot application. We describe the use of orthogonal methods in which the specificity of an antibody in a particular application is determined based on correlation of protein or RNA abundances for the target across different samples using antibody- independent methods (mass spectrometry and RNA-seq). We show that pairwise correlation between expression levels can be used to evaluate and score the specificity of different antibodies in a standardized manner. In this study, we investigated both proteomics and transcriptomics methods for validation of antibodies for Western blot applications and compared these methods with a genetic approach based on siRNA knockdown. The result show that both orthogonal methods are highly suitable for validation of antibodies and that they could be used to investigate on- and off-target binding for antibodies with multiple bands present in the Western blot assay. This approach has the potential for standardized high-throughput validation of thousands of antibodies for Western blot application using a well-characterized cell-line panel.

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Thermal profiling of Breast cancer cells reveals proteasomal activation by CDK4/6 inhibitor Palbociclib

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Palbociclib is a recent drug approved by the FDA for phase III clinical trials in treating estrogen-receptor-positive and HER2-negative breast cancer. In vitro, palbociclib, a selective inhibitor of CDK4/6, was shown to reduce cellular proliferation of breast cancer cell lines by blocking progression of cells from G1 into S phase of the cell cycle. While the primary targets of palbociclib have been deciphered, the molecular mechanisms leading to off-targets effects and drug resistance are not known. To identify new palbociclib protein targets we applied a Cellular Thermal Shift Assay and proteomic analysis (MS-CeTSA) that works under the assumption that protein-drug interaction stabilises proteins thus leading to an increase in thermostability. MCF-7 breast cancer cells were cultured under palbociclib treatment and heated from 37°C to 68°C. Supernatants from 10 different temperatures were collected for quantitative proteomic analysis using Tandem Mass Tag. The calculated fold changes, as a function of temperature, follow a sigmoidal trend reflecting the thermal stability of proteins and their disappearance with increasing temperature. Proteomic analysis on the Orbitrap Fusion allowed the quantification of 38,498 peptides corresponding to 5,516 proteins. As expected, CDK4/6, the molecular targets of palbociclib were among the 10 most-changing proteins with a temperature shift (Δtm) of 4.9°C and 3.7°C. Validation by WB-CeTSA confirmed CDK4/6 stabilisation. Classification of identified protein kinases according to the calculated Δtm revealed new potential targets such as CAMK2D, AKT1 and MTOR proteins. Preliminary validation in-vitro indicated that the MTOR-PI3K signalling pathway may be impaired by the action of palbociclib. In addition, the MS-CeTSA also revealed a stabilisation of several proteasomal proteins of the 20S core proteasome through the inhibition of the proteasome-associated scaffolding protein ECM29. Taken together, these data suggest that off-targets effects during palbociclib treatment positively participate by blocking tumor progression from G1 into S phase of the cell cycle.

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Benchmarking statistical approaches for label-free quantitative proteomics

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Background
Quantitative proteomics studies use MS data to compare samples to discover up or down-regulated proteins. Statistical techniques are required to determine which proteins are changing significantly in abundance, beyond replicate to replicate variation, however the key assumption of standard parametric testing (t-test; ANOVA) cannot necessarily be made in proteomics experiments. Large numbers of features with small numbers of samples, produces large sample-to-sample variability, where mean comparison can be distorted by outliers. Several alternative statistical approaches have been proposed. This study assesses their accuracy through benchmarking using datasets where underlying ratios of proteins across samples are known.

Methods
Four statistical packages were assessed by their ability to detect relative quantitative changes in controlled experiments, based on linear-mixed modelling, Bayesian hierarchical modelling, robust ridge regression and Student's t-test. Benchmarking datasets with known protein concentrations spiked into an unchanging background allows pairwise comparison simulating fold-changes. Proteins correctly identified as differentially expressed and incorrectly detected background proteins were counted to calculate true and false positives.

Results
Sensitivity was plotted against Q-value to give pseudo ROC plot which was used for evaluation. Area under the curve closest to 1 indicates the best performance, with maximum sensitivity achieved without false positives. Optimal performance requires a particular combination of protein inference method, normalisation and statistical testing.

Conclusions
Our results demonstrate the differences introduced by each step of a quantification pipeline peptide to protein inference, normalisation and statistical testing, to provide community guidelines for optimal processing of proteomics data.

Keywords
Label-free; differential expression; statistical analysis.

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Landscape of proteomic alterations in cervical carcinomas through laser microdissection sampling.

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Background
Cervical cancer is characterized by a well-defined pre-malignant phase, cervical intraepithelial neoplasia (CIN). Identification of high grade CIN lesions by population-based screening programs and their subsequent treatment has led to a significant reduction of the incidence and mortality of cervical cancer. Cytology-based testing of cervical smears is the most widely used cervical cancer screening method, but is not ideal, as the sensitivity for detection of CIN2 and higher (CIN2+) is only ~55%. Therefore, more sensitive and specific biomarkers for cervical cancer and its precancerous stages are needed.

Methods
We compared an equivalent of 8000 tumor, normal epithelium and stroma cells of frozen cervix tissue from 22 patients with cervical squamous cell cancer (early- and late stage, each stage 11 samples) and 13 healthy subjects obtained by laser capture microdissection. The proteins were enzymatically digested into peptides and measured by high-resolution mass spectrometry (Orbitrap Fusion, Thermo Fisher Scientific). Identified proteins were visualized by Scaffold Proteome software and the Bonferroni correction was applied to adjust the p-value for multiple testing within the different groups. Results were loaded into the Ingenuity Pathway Analysis to assign proteins to different network interactions.

Results
By comparing this set of 11 early-stage, 11 late-stage cervical squamous cell cancer tissue with epithelium and stroma cells from 13 healthy subjects, 30 significant differentially expressed proteins were found after correction for multiple testing (Bonferroni correction). Among these 30 proteins the MCM protein family was strongly up-regulated. By Ingenuity Pathway Analysis we assigned 17 from the 30 up-regulated proteins to a network that is related to DNA replication recombination, repair and cellular development.

Conclusions
We assume that up-regulation of MCM proteins is critical for tumor progression and might serve as viable targets for anti-cancer therapy as well as molecular markers for diagnosis of cervical cancer.

Keywords
Proteomics of cervical carcinoma.

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Extracellular Matrix Function Using Integrated Omics Dissect Cell Wall Mediated Immune Dynamics in Wilt disease

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Background

Cell wall integrity signaling orchestrates complex regulatory mechanism that control mechano-sensing and turgor-driven deformation within the dynamic milieu of extracellular matrix (ECM) dictating cell fate decisions during patho-stress. Wilt disease caused by hemibiotrophic fungus Fusarium oxysporum is a major impediment for global crop productivity. Regulatory behavior of cells using integrated OMICs may reveal complex architecture of living systems, including disease and immune response. To elucidate the role of ECM and molecular mechanism associated with cellular immunity, the temporal changes of ECM proteome and metabolome was studied in wilt susceptible chickpea (Cicer arietinum) cultivars upon F. oxysporum infection.

Methods

Patho-stress was imposed on three-week-old seedlings and tissues were harvested at different post-infection time points. Temporal proteome and metabolome was developed with ECM enriched fraction using quantitative iTRAQ coupled Triple-TOF/MS and GC-MS analyses, respectively. Integrated global network was built to identify disease and immunity related pathways. Furthermore, qRT-PCR analysis was performed to validate the omics datasets.

Results

Differential display of Fusarium infected resistant and susceptible chickpea cultivar proteomes revealed 82 immune-responsive proteins (IRPs) and 92 patho-stress responsive proteins (PSRPs) presumably associated with wall mechanics, acidification and innate defense. Furthermore, wilt-responsive metabolome profile displayed significant alteration in metabolites associated with global metabolic pathways, particularly phenyl-propanoid and suberin paralleling the proteomic analysis. Network analysis identified major protein hubs enriched in known and novel disease- and immunity-related prognostic proteins pointing towards the onset of disease signaling and metabolic pathway activations. Multivariate and network-based analyses successfully revealed the difference between the covariance structures of the integrated data sets.

Conclusions

Integrated multi-omics analysis for the first time provides an insight into the complex network operating in the ECM and impinges on the surveillance mechanism of plant innate immunity. The identified novel prognostic biomarkers have great potential for metabolic pathway bioengineering in future crop improvement program.

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A novel MS-cleavable tag for the analysis of redox-sensitive cysteines by shotgun proteomics

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Background: Accumulating evidence suggests that reactive oxygen and nitrogen species are not only toxic molecules but can act as second messengers and modulate intracellular signaling by reversible cysteine oxidation. Various approaches have been developed to covalently label and biochemically enrich redox-sensitive cysteines prior to their analysis by mass spectrometry (MS). However, proteomics strategies that do not require enrichment steps would be highly desirable for the sensitive and large-scale characterization of cysteine modifications.
Methods: We developed a novel MS-cleavable enrichment tag that covalently reacts with thiols. Tag-coupled proteins were digested and peptides were analyzed with UHPLC and high resolution MS/MS. Low energy all ion fragmentation generated neutral losses of labeled peptides and allowed the specific selection of these ions for MS2 scans. We applied our technology to label and identify oxidation-sensitive cysteines in HEK 293T cell lines with deficiencies in antioxidant enzymes generated by CRISPR/Cas9.
Results: We show that the median collision energy required to cleave the MS-labile site of our tag is below the median energy required for peptide backbone fragmentation. Neutral loss-dependent acquisition allowed the specific identification of tagged peptides without prior biochemical enrichment and increased the reproducibility of quantification between samples. Using this approach, we identified common and specific targets of antioxidant enzymes such as superoxide dismutases and thioredoxins.
Conclusions: We present a chemical proteomics strategy based on a new MS-cleavable tag to analyze cysteine oxidation without prior biochemical enrichment. This technology allows the generic identification of the molecular targets of antioxidant enzymes and of redox-regulated cysteines in intracellular signaling cascades. Furthermore, the MS-cleavable moiety enables the engineering of tags for the selected analysis of covalently-labeled peptides with other post-translational modifications.

Keywords: Redox proteomics, cysteines, CRISPR/Cas9, neutral loss-dependent acquisition

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Nobel protein interaction analysis method: MIK-MS (Molecular Interaction and Kinetics Mass Spectrometry)

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Background

For drug discovery, protein-protein or protein-small to intermediate molecule interaction have been screened mainly with the Surface Plasmon Resonance (SPR). We have developed a novel method using nano Pore Optical Interferometry (nPOI) that is similar with SPR. We also have determined the screened molecules simultaneously by connecting the nPOI to mass spectrometry, which is an advantage of nPOI over SPR. nPOI can measure the association and dissociation rates of biomolecules without labels. Since nPOI has a larger amount of ligand capture capacity than SPR, it can be used to detect analytes directly using a Mass spectrometry. We named this integrated system as Molecular Interaction Kinetics- Mass Spectrometry (MIK-MS). MIK-MS would be a powerful tool to screen drug candidates.

Methods

To the sample channel chip, Carbonic Anhydrase II (CAII) is immobilized using the standard amino coupling. The reference channel is a bare chip. The interaction kinetics of the small molecules Sulpiride, Furosemide, Dansylamide, Acetazolamide, 4-Carboxylbenzene-sulfonamide, and Sulfanilamide were investigated separately or simultaneously. Eluted fractions from the sample channel were measured using a MS.

Results

Using MIK-MS, it was confirmed that each compound can be quantitatively detected with high sensitivity from eluted fractions as association and dissociation of compounds was easily distinguished. Almost compounds showed similar results to SPR, and each compound could be identified by its m/z.

Conclusion

MIK-MS can analyze the interaction between proteins and compounds in a mixture. Therefore it is expected to be an important tool for biomarker and drug discovery.

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Studying the effect of food systems on gut epithelium cells analyzed by LC-MS proteomics

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Background

DuPont Nutrition Biosciences ApS, produces various food ingredients including protein hydrolysates, prebiotics, probiotics and other live cultures for both humans and animals. As part of the R&D process, DuPont are interested in the effect on the gut epithelium of a given product. An experimental setup has been designed were control samples and human fecal samples are tested on CaCo-2 cells in vitro to model the gut barrier.

Methods

CaCo-2 cells were grown on transwell inserts into a mono cell-layer. The integrity of the monolayer was controlled using the trans-epithelial electrical resistance (TEER). Fecal samples were diluted in PBS and sterilized through a 22µm filter. Fecal samples were added to the apical side of the transwell and incubated for 24-hours. Cells were washed and harvested for LC-MS/MS based proteomics after 24-hour stimulation. Samples were lysed and digested following the FASP procedure. A total of 52 sample were analyzed using a 2-hour gradient on a nanoflow-UHPLC connected to an Orbitrap Fusion. DIA data was obtained using 27 variable DIA windows and analyzed in Spectronaut 10.

Results

In total 7,895 protein groups, out of 11,163 in the spectral library, were quantified using the DIA label-free quantification. Statistical analysis showed that 223 proteins were significantly regulated between fecal samples and control samples at 5% FDR. Pathway analysis of regulated proteins in IPA, showed that several pathways were affected including cell cycle control, uptake by invagination and endocytosis.

Conclusions

The DIA proteomic analysis could reproducibly quantify a high number of proteins in the CaCo-2 cell digests. Results from such screening system creates valuable new insights into how food products effects a cell line on mechanisms that are associated to obesity and other metabolic issues.

Keywords: Food ingredients, CaCo-2 cells, label-free quantification.

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In-depth analyses of protein abundance, phosphorylation, synthesis and degradation during neuronal development and synaptic plasticity

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Background
Many human neurological disorders remain poorly understood due to lacking knowledge of the underlying molecular mechanisms, where brain developmental processes seem critical in acquiring the disease. Therefore, we first investigated neuronal differentiation in cultured hippocampal neurons at different stages and monitored changes in protein abundance. Next, we assessed a form of synaptic plasticity in these hippocampal neurons, named long-term depression (LTD). Dysfunction of LTD is implicated in neurodevelopmental disorders like autism and the major mechanism relies on the rapid and local synthesis of new proteins in dendrites. Thus, defining the regulation and dynamics of protein synthesis and degradation are essential to better understand these plasticity mechanisms.

Methods;
For newly synthesized proteins, depleted medium was supplemented with L-azidohomoalanine (AHA), heavy lysine and arginine. Newly synthesized proteins were enriched using click-chemistry. Proteins were digested and phosphorylated peptides were enriched using Fe(III)-IMAC using the AssayMAP Bravo Platform (Agilent). Samples were analyzed by high-resolution nanoLC-MS/MS.

Results;
We identified 6,753 proteins, covering crucial neuronal developmental processes including axon outgrowth, dendrite formation and synaptogenesis. To highlight the strength of our data, we focused on neural cell adhesion molecule 1 (NCAM1) as a regulator for dendritic outgrowth in neuronal development. Biochemical validation suggest that NCAM1 stimulates dendritic arbor development by promoting actin filament growth at the dendritic growth cone. Next, we focused on synaptic plasticity, requiring extremely sensitive enrichment strategies, since typical protein recovery from primary neuron cultures is around 50ug. Therefore, first we optimized our phosphopeptide enrichment; enabling the identification of 6,095 unique phosphopeptides and adapted an AHA/pSILAC approach, identifying 1,200 newly synthesized proteins. In this study we identified several kinases with important roles in LTD and identified changes in the AMPA receptor endocytosis pathway.

Conclusions;
This study revealed novel insights into neuronal development and molecular mechanisms underlying LTD.

Keywords
Phosphoproteomics, synaptic plasticity, translation, degradation,

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Quantitative proteomic analysis of vitreous humor from idiopathic epiretinal membrane and macular hole patients

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Background: Age-related vitreoretinal interface diseases idiopathic epiretinal membrane (iERM) and macular hole (iMH) are major potentially vision-threatening vitreoretinal public health issues affecting millions of aging people globally. Deeper understanding of biomechanical mechanisms and signalling pathways involved in these eye diseases is warranted. So far, proteomes of iERM and iMH remain largely unstudied.
Methods: Human vitreous is a transparent extracellular matrix gel which contact with the retina. Because of this close interaction, the physiological and pathological conditions of the retina affect the protein components in the vitreous. Here, we quantitatively mapped the vitreous proteome changes in iERM (n=26) and MH (n=21) patient samples using LC-MS based label-free quantitative proteomic analysis. Seven diabetic retinopathy patients with macular edema (DME) were included in analysis as control group.
Results: In total, we could quantify 936 vitreous proteins. The most common proteins in every sample group were highly similar, consisting of high abundance proteins such as serum albumin, complement factors and apolipoproteins. When the iERM and iMH proteomes were compared to the vitreous proteome of DME, a clear difference was detected. The majority of proteins that were more abundant in iERM and MH group were linked to cell adhesion and ECM organization or nervous system development. Also specific signaling proteins were more abundant in iERM and MH samples.
Conclusion: Thorough large-scale MS-based quantitative proteomic analysis revealed that neurodegeneration rather than neuroinflammation, seems to play an important role in the pathogenesis of iERM and MH. The identification of the key signalling pathways and novel vitreous biomarkers of the iERM and iMH eye diseases is critical since bringing novel insight into these pathological conditions as well as for the potential development of new therapeutic molecules and for the new use of existing drugs is needed.
Keywords: epiretinal membrane, vitreous humor, label-free quantitative proteomics, neurodegenetarion, Wnt signaling

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Mining drug resistant targets from non-small cell lung cancer by phosphoproteomics

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Background
EGFR-tyrosine kinase inhibitor (TKI) has demonstrated improvement in survival of patients with EGFR mutation including exon19 deletions (del19) and exon21 (L858R) in non-small cell lung cancer (NSCLC). However, patients eventually acquire resistance and recurrence. The drug resistance is generally triggered by secondary mutation of EGFR, MET and HER2 amplification. However, clear mechanisms of drug resistance are yet to be discovered. Thus, we attempted to clarify the mechanism and targets contributing to EGFR-TKI sensitivity and resistance in NSCLC by a quantitative phosphoproteomic approach.
Methods
We used a clinically relevant model to study acquired gefitinib resistance in NSCLC cells. The cells were treated with gefitinib, lysed, digested by tryptic digestion, and labeled by iTRAQ-8plex. The phosphopeptides were enriched by IMAC stage-tip, analyzed by LTQ-Orbitrap Fusion, and processed by PD2.1. The differential phosphoproteins were further validated by Western blot and functional assay.
Results
Upon gefitinib treatment of the TKI-sensitive cells for 1 and 3 days, western blot showed significantly deactivation of pY1068-EGFR and its downstream ERK. In the resistant cells, however, we observed persistent phospho-ERK signal upon gefitinib treatment, suggesting that TKI resistance is EGFR-independent in the resistant cells. As expected, gefitinib led to down-regulation of the EGFR-pY1197), ERK1/2-pT202 within the known TKI-responsive canonical NSCLC pathway. However, the resistant mechanism with phosphorylation signaling cascade in NSCLC is still puzzled. Among these 3933 quantified phosphopeptides, 150 phosphopeptides showed up- or down-regulated phosphorylation level in gefitinib-treated sensitive cells, while no change in resistant cells. These include known resistant targets, PXN has been reported to confer resistance to gefitinib in NSCLC. The potential drug-resistant phosphoproteins will be evaluated their potential roles in regulating TKI resistance in NSCLC cancer therapy.
Conclusions
The quantitative phosphoproteomic approach reveals the gefitinib-responsive and resistant phosphorylation network, which may shed light on the resistant mechanism for NSCLC.
Keywords
NSCLC, phosphoproteome, TKI-resistance

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Proteoforms of transthyretin - candidate biomarkers in diagnosis of Obstructive Sleep Apnea

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Background: Obstructive Sleep Apnea (OSA) is considered a cardio-metabolic disorder, which diagnosis at early stage is still challenging. Recent studies found association between transthyretin (TTR) protein modifications present in plasma and sleep disorders (1,2). Mass Spectrometric Immunoassay (MSIA) was successfully applied previously in the identification and quantification of TTR variants (3). We took advantage on this top-down proteomics method to investigate TTR proteoforms as potential blood-based biomarkers for screening subjects at OSA risk.

Methods: Cohort of 68 male patients with suspected OSA underwent night laboratory-based polysomnography (PSG) diagnosis for OSA. Blood from all patients were taken for biochemical and proteomic analysis. MSIA with TTR specific immunoaffinity columns was used to perform individual analysis of plasma samples (3). Data analysis was performed using Flex analysis (Bruker). Statistical analysis was carried-out in R4 (Kruskal-Wallis, p-value <0.10, followed by Dunn´s test).

Results: According to PSG-respiratory disturbance index (RDI), patients were classified into four groups: control snorers (n=16), mild (n=27), moderate (n=10) and with severe OSA (n=15). MSIA analysis identified six proteoforms of transthyretin present in plasma samples: native (TTRn), oxydated (TTRox), sulfonated (TTRs), cysteinylated (TTRcys), cysteine-glycine (TTRcys10gy) and cysteinyl-glycine (TTRcysgly). With exception of TTRs and TTRox, all identified proteoforms were significantly (p-value<0.05) downregulated in OSA (average values in severe OSA were half of control average values). These findings were in concordance with severity of the OSA syndrome.

Conclusions: MSIA results from training set imply that analysis of plasma TTR proteoforms may be of use to distinguish subjects with OSA from snorers, especially highlighted for patients with severe OSA syndrome. These results are in concordance with RDI findings obtained from polysomnography, a gold standard in diagnosis of OSA. Pathophysiological mechanisms underlying these changes on plasma TTR need further investigations.

Keywords: Mass Spectrometric Immunoassay, obstructive sleep apnea, transthyretin, proteoforms, biomarker

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Mass spectrometry characterization of DOTA-Nimotuzumab as innovative β- tracer in radio-guided surgery

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Background
Some researchers of our group developed an innovative β- radiations probe for its applications in radio-guided surgery. Monoclonal antibodies labelled by radionuclides throughout a bifunctional chelating agent are good candidates for the development of new radiotracers. Among them, Nimotuzumab modified by para-S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraaza cyclododecanetetraacetic acid (p-SCN-Bn-DOTA) has been proposed as vector of β– radiations as it targets the extracellular domain of epidermal growth factor receptors, which are highly expressed in several cancers. In this work we described the characterization of p-SCN-Bn-DOTA-Nimotuzumab immunoconjugates through a comprehensive mass spectrometry proteomic platform. Its detailed description is relevant for future clinical applications.

Methods
Characterization of Nimotuzumab and p-SCN-Bn-DOTA-Nimotuzumab intact masses was achieved by SDS-PAGE, MALDI-TOF and ESI-MS flow injection analyses. Nimotuzumab underwent to steps of deglycosylation, reduction, alkylation and reverse-phase liquid chromatography in order to separate light and heavy chains. Purified chains were digested enzymatically (trypsin, pepsin, chymotrypsin and thermolysin) and the resulting peptides were characterized by HPLC-ESI-MS/MS for the assignment of lysine residues involved in the binding with p-SCN-Bn-DOTA.

Results
SDS-PAGE, ESI flow injection and MALDI-TOF experiments showed how the entire Nimotuzumab has been modified by the reaction with p-SCN-Bn-DOTA. From these analyses we detected up to 3 p-SCN-DOTA for the light chain, while up to 12 for the heavy chain, for a total of 30 p-SCN-Bn-DOTA linked to Nimotuzumab. By means of the enzymatic digestions, we identified a partial number of p-SCN-Bn-DOTA modified peptides.

Conclusions
This work represents another step in the challenging world of monoclonal antibodies characterization. We were able to establish the number and the position of lysines binding p-SCN-Bn-DOTA. This information is strongly important for the development of a new biopharmaceutical compound that in the next future could be used in coupling with the pioneering β- probe for evidencing residual neoplastic tissues during surgery.

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Glucagon was identified as potential therapy target in colorectal cancer through label-free quantitative proteomic analysis

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Abstract:

Background：Colorectal cancer is the fifth common cancer in developing countries. It is urgent to identify novel biomarkers and therapeutic targets with proteomics approach. In this study, we aimed to identify novel biomarkers involved in colorectal cancer and provide potential therapeutic targets through a label free quantitative proteomics approach.

Methods：
Six Pairs of colonrectal tumor tissue and adjustment normal colonrectal tissues were obtained from surgical resection and analyzed with QExactive Plus coupled with nanoLC system with a label-free quantitative proteomics approach. The differentially expressed proteins were validated by Western blot analysis. The calcium regulation network was predicted from differenctially expressed proteins with Ingenuity IPA. The calcium signaling network were further investigated with total internal reflection (TIRF) microscopy .

Results ：

A total of 4,266 proteins were identified and quantified. Among them, 136 proteins presented a significant expression difference. Western blot confirmed GTP ,PGK,ATP5B,CAPG,ANXA4 and MAPK14 are overexpressed in the cancer tissue. A group of proteins including CALR ,ANXA6 ,ATP5A ,CRMP2 ,Aactin ,CAMKII ,and Glucagon were declined in the colonrectal cancer . We further confirmed the low expression of GCG in 40 pairs of colorectal cancer tissues using immunohistochemical analysis, and found that the expression levels of GCG were correlated with tumor development stages and metastasis. The cancer therapy potential of glucagon receptor agonist was further
Validated with TIRF microscopy.

Conclusion：

Our findings suggest that calcium regulating network plays a critic role in colorectal cancer. Glucagon will be a promising clinical biomarker in colorectal cancer and GLP-1 could be a potential therapy target.

Keywords: Colorectal Cancer, Quantitative proteomics, GLP-1, Biopsy

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Integrative proteo-metabolomic study illustrates role of nucleus in blast disease signaling and host-specific resistance

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Background

Nucleus, the regulatory hub is a dynamic system that regulates protein expression and serves as modulator of signaling events dictating cell fate decisions during patho-stress. Blast disease caused by hemibiotrophic fungus Magnoporthe grisae is a major impediment for global crop productivity. Regulatory behavior of cells using integrated OMICs may reveal complex architecture of living systems, including disease and immune response. To elucidate the role of nucleus and molecular mechanism associated with cellular immunity, the temporal changes of nuclear proteome and metabolome was studied in blast resistant and susceptible rice (Oryza sativa) cultivars upon M. grisae infection.

Methods

Patho-stress was imposed on three-leaf-stage seedlings and tissues were harvested at different post-infection time points. Temporal proteome and metabolome was developed with nuclear enriched fraction using quantitative 2-DE and iTRAQ coupled ESI-MS/MS and Triple-TOF/MS and GC-MS analyses, respectively. Integrated global network was built to identify disease and immunity related pathways. Furthermore, qRT-PCR analysis was performed to validate the omics datasets.

Results

The differential display of Magnoporthe infected resistant and susceptible rice cultivar proteomes revealed 215 immune-responsive proteins (IRPs) and 150 patho-stress responsive proteins (PSRPs) presumably associated with nucleic acid biogenesis and chromatin remodeling. Furthermore, blast-responsive metabolome profile displayed significant alteration in 165 (IRMs) and 198 (PSRMs) metabolites associated with global metabolic pathways, particularly sugar alcohol and organic acids paralleling the proteomic analysis. Network analysis identified major protein hubs enriched in known and novel disease- and immunity-related prognostic proteins pointing towards the onset and context of disease signaling and metabolic pathway activations. Multivariate and network-based analyses successfully revealed the difference between the covariance structures of the integrated data sets.

Conclusions

Combined analyses of multi-omics landscape of rice not only provide useful insights into the underlying mechanism of blast resistance, but also enlist novel biomarkers for targeted genetic manipulation for food and nutrition security.

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Glycoproteomic and glycomic analysis of human induced pluripotent stem cells and stem cell-derived cardiomyocytes

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Background:
Human induced pluripotent stem cells (hiPSCs) and hiPSC-derived cardiomyocytes are promising tools for future regenerative medicine approaches. Importantly, for application in patients, the generated cells have to be well purified and characterized by using specific markers, typically protein or glycan structures. To date, however, only few cell surface markers for cardiomyocytes are known. The aim of our study is to overcome this scarcity of markers by characterizing the glycoproteome and the N-glycome of hiPSCs, cardiomyocyte progenitors and cardiomyocytes derived thereof.

Methods:
For glycoproteomics, we enriched for cell surface sialo-glycoproteins using the PAL (periodate oxidation and aniline-catalyzed oxime ligation) technique in combination with SILAC (stable isotope labeling by amino acids in cell culture). Analysis of the N-glycome was performed by CGE-LIF (capillary gel electrophoresis coupled to laser-induced fluorescence).

Results:
The glycoproteomics approach identified 880 proteins in a comparison of hiPSCs harvested at d0, d7 and d15 of differentiation. According to gene ontology analysis, plasma membrane proteins were strongly enriched by PAL, comprising ~47% in the PAL-labeled sample versus ~24% in a whole cell proteome analysis. All so far known cardiomyocyte cell surface markers, i.e. ALCAM (Rust et al., 2009), EMILIN2 (Van Hoof et al., 2010), SIRPA (Dubois et al., 2011; Elliott et al., 2011) and VCAM1 (Uosaki et al., 2011), could be reconfirmed using our approach. Moreover, a number of so far uncharacterized proteins were found to be significantly regulated and thus represent promising candidates as novel cell surface markers for cardiomyocytes.
The N-glycome analysis showed a significant decrease in antennae fucosylation and beta-1,3-linked galactose residues along with an increase of bisecting GlcNAc structures in differentiated cardiomyocytes compared to undifferentiated hiPSCs.

Conclusions:
By combining glycoproteomics and glycomics we present a comprehensive dataset enabling further identification of novel cell type-specific markers.

Keywords:
Glycoproteomics, glycomics, pluripotent stem cells, cardiomyocytes, cell surface markers

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Boiling produces hypoallergenic cashew nuts with potential to safely desensitize affected patients.

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Background

In Australia, cashew nut is the second most prevalent nut allergy behind peanut, with no approved treatment available. We have previously demonstrated that boiling peanuts reduces the allergenicity of peanuts by 8-fold at 2 hours and 19-fold at 12 hours. T-cell reactivity was unaffected. Boiling leaches allergens from the nuts, denatures and fragments the allergens while allergen peptides are retained in the peanuts. We have subsequently tested the clinical utility of boiled peanuts by treating a small cohort of peanut allergic children with the hypoallergenic peanuts followed by roasted peanuts. Hypoallergenic peanuts resulted in a low rate of mild allergic reactions and permitted the subsequent complete desensitization with roasted peanuts, also with few mild allergic reactions. We have now tested whether boiling also reduces allergenicity of cashew nuts.

Methods
The effect of boiling cashew nuts for up to 12 hours on cashew nut allergens was investigated by 1D/2D electrophoresis. Reduced allergenicity was determined by western blot, inhibition ELISA and by in vivo patient skin prick tests. The presence of fragmented allergen peptides was monitored by qTOF mass spectrometry.

Results
Boiling for 2 hours reduced IgE-reactivity by 10-fold and skin prick test (SPT) wheal diameter by 54% while boiling for 12-hours reduced IgE reactivity by >32-fold and SPT wheal diameter by 75%. Leaching and fragmentation of allergens was observed with retention of peptides from the allergen Ana O 2.

Conclusions
Boiling cashew nuts reduces the IgE reactivity greater than that seen for peanut extracts at the equivalent time point. Peanut extracts contain Ara h 2, a heat-resistant allergen which is likely to account for the differences. Hypoallergenic boiled cashew nuts to initiate desensitization followed by allergic cashew nuts has potential as a safe treatment for cashew nut allergy.

Keywords
Cashew nut allergy
Desensitization
qTOF mass spectrometry

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Secretome Analysis of BSA-free Cardiomyocyte Differentiation

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Background:
Cardiovascular diseases cause a high number of deaths worldwide. The heart has a very low regeneration capacity and organs for transplantation are scarce. Therefore regeneration of lost myocardium with stem cell-derived cardiomyocytes is an attractive strategy for regenerative medicine. Human pluripotent stem cells (hPSCs) can be differentiated into cardiomyocytes but the molecular mechanism behind that differentiation is poorly understood. Differentiating cells secrete autocrine/paracrine factors into the supernatant functioning as important regulatory determinants. A very efficient protocol leading to high proportions of cardiomyocytes from hPSCs is based on medium supplemented with B27. However, this B27 supplement contains high levels of bovine serum albumin (BSA). Considering that secreted factors are found at concentrations in the range of ng/ml, the enormous excess of BSA in cell culture supernatants hampers their mass spectrometry-based identification. Therefore the development of BSA-free cardiomyocyte differentiation conditions is pivotal for the subsequent MS-based analysis of secreted proteins.
Methods:
We generated a custom-made B27 and omitted the BSA. hPSCs were differentiated over 15 days in which cell culture supernatants of 7 different time points were analyzed for secreted factors by a label-free quantification mass spectrometry approach.
Results:
hPSCs could be successfully differentiated into cardiomyocytes under BSA-free conditions. Overall, more than 5,000 proteins were identified and their relative levels at the different time points were quantitatively assessed. One part of the identified proteins includes factors involved in signaling pathways that are related to cardiomyocyte development, like Wnt- or TGFβ signaling. Extracellular Matrix (ECM) proteins like collagens and ECM-modifying enzymes were identified as well. We further detected several cytokines with not yet determined function on cardiomyogenesis like interleukins, complement factors, BMPs or thrombospondins.
Conclusion:
Some of these highly secreted factors might improve the cardiomyogenic differentiation of hPSCs for approaches in regenerative medicine.
Keywords: secretome, cardiomyocytes, human pluripotent stem cells

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Post-translationally modified alpha-1-microglobulin as a plasma biomarker for the early diagnosis of Parkinson's disease.

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Background: Parkinson's is a progressive neurodegenerative disease that affects 1% of the population over the age of 60. Diagnosis of idiopathic Parkinson's Disease is difficult and there are no molecular imaging or biochemical based test available to aid in the diagnosis. Confirmation of diagnosis can only occur at autopsy. A diagnostic set of tools would be a highly significant advance and assist in the recruitment of subjects for clinical trials.

Methods: We have used plasma samples from the Victorian Parkinson's Disease registry and specific protein enrichment strategy to conduct a proteomic screen for plasma biomarkers of Parkinson's (n=94). We also conducted a quantitative LC-MS analysis of 44 plasma metabolites in Parkinson's, Alzheimer's and control subjects (n=255).

Results: We found that a postranslationally modified version of the alph-1-microglobulin (A1M) was significantly elevated plasma from individuals diagnosed with idiopathic PD. The diagnostic accuracy of this marker was greater than 85%, indicating great potential for clinical utility. We validated our discovery by measuring the levels of modified A1M in samples from newly diagnosed cases of PD. We found that the A1M and metabolite biomarkers found in our discovery phase were significantly elevated in the newly diagnosed individuals indicating that standard pharmacological treatment does induce modification of the A1M protein biomarker.

Conclusions: The elevated levels of A1M and metabolites in Parkinson's disease plasma have greater than 85% sensitivity and specificity to detect the neurodegenerative process. Further we demonstrated that the biomarkers are elevated in newly diagnosed Parkinson's disease cases which implies that the markers were elevated before they presented at the clinical with symptoms. Our results indicate that a blood-based test for the early detection of PD could form the basis of a diagnostic screen to detect PD at the earliest stage possible.

Keywords: Blood, biomarker, 2D gel proteomics, Parkinson's Disease, Neurodegeneration

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Label-free differential phosphoproteomic analysis of recombinant Chinese Hamster Ovary cells reveals growth related phosphoproteome modifications.

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Background
The Chinese hamster ovary (CHO) cell line is the animal cell expression system of choice for production of recombinant biotherapeutics; as a result there is significant scientific and commercial interest in research that could improve the productivity of CHO cells. Phosphorylation is one of the most important post-translational modifications, playing a crucial role in regulating many cellular processes. However, its role in regulating CHO bioprocess-relevant phenotypes has not been studied in detail to date. In CHO cell cultures, the phosphoproteome will change over different phases of growth and these modifications can give crucial indications on the cellular state in terms of growth, viability and productivity. We applied mass spectrometry based phosphoproteomics in recombinant CHO cell cultures to monitor changes in the expression patterns of the phosphoproteome during lag, exponential, and stationary phases of the growth curve.

Experimental approach
CHO DP12 cells were cultured in serum-free media at 37ºC. Samples were collected at the different growth phases, i.e. lag, exponential, stationary and death. Phosphopeptide enrichment was performed by MOAC (TiO2) chromatography in parallel with IMAC (Fe-NTA) chromatography, prior to quantitative label-free LC-MS/MS phosphoproteomic analysis. Differentially expressed phosphopeptides were identified using Progenesis QI for Proteomics. PhoshoRS filters were set at >75% for assignment of site-specific phosphorylation. Gene Ontology was carried out using DAVID functional annotation software.

Results
Phosphoproteomic comparisons between the different growth phases revealed differential phosphorylation of various proteins, including those with potential regulatory significance such as transcription factors, cell cycle modulators and key signalling molecules. These results provide a functional understanding of the CHO cell phosphoproteome during growth in suspension culture.

Conclusion
Overall, this study demonstrates the potential benefits of combining phosphopeptide enrichment strategies, high resolution LC-MS/MS and label-free differential analysis for large sample sets, in order to achieve a comprehensive understanding of the phosphoproteome of CHO cells.

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Assessment of the activation of macrophages by quantitative secretome analysis

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Background

The secretome of immune cells provides a promising resource for discovery of specific molecular markers and targets for pharmacological intervention. A number of studies have established that statins are effective in the primary and secondary prevention of coronary artery disease (CAD), driving its pleiotropic biological actions in different cells types through various mechanisms, including the inhibition of protein prenylation of a variety proteins related to modulation of oxidative stress, inflammation, immune response, apoptosis and thrombosis. However the precise mechanisms underlining these actions and their consequences in cellular communication remain largely unknown.
The aim of the study was to develop a methodology for the analysis of macrophage activation by secretome quantitation and to assess the effect of atorvastatin on RAW264.7 macrophages stimulation.

Methods

Differential protein secretion evoked by atorvastatin pre-treatment (300 nM) in stimulated (200 ng/ml LPS, 12 hours) RAW264.7 macrophages was assessed by iTRAQ quantitation of cell culture media (secretome).

Results

The identification of 7069 unique peptides (FDR < 1%) enabled for the quantitation of 884 proteins secreted by RAW264.7 macrophages upon LPS activation of TLR4 receptors. Among them 32 and 29 were statistically significantly up- and down-regulated, respectively. Atorvastatin treatment resulted in several interesting protein abundance changes, including those related to inflammation (TNFα), oxidative stress (i.a. metallothioneins, ferritins, thioredoxin, SOD), immune response (i.a. plastin-A, prosaposin, cathepsins B,S,Z) and proteolysis (incl. prolyl endopeptidase, Xaa-Pro dipeptidase).

Conclusions

Proteomic iTRAQ approach allows for the qualitative and quantitative exploration of the macrophage sercetome at once and points to several proteins with anti-inflammatory, anti-oxidative and immunomodulatory activities of atorvastatin in activated RAW264.7 cells. Determination of exact macrophage secretome compositions will aid to understand the pleiotropic molecular mechanisms of statins action and disclose potential targets for the design of new pharmacotherapies.

Keywords

secretome; macrophages; statins; iTRAQ

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Optimization of Biotin Enrichment Protocols for the Analysis of Biotinylated Proteins by LC-MS/MS

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Background
Interaction between biotin and avidin is used widely for the targeted enrichment of proteins from complex mixtures. The recovery of biotinylated proteins from beads and identification of specifically enriched proteins remain major challenges. Here, we compare different strategies for analysis of enriched biotinylated proteins and assess their performance and specificity.

Methods
For evaluation of unspecific binding, SILAC human embryonic kidney (HEK) cells were mixed, lyzed and incubated with streptavidin beads. After washing proteins were on bead digested and peptides fractionated by offgel electrophoresis. For method comparisons light SILAC labeled HEK cells were stable transfected with a mutated version of the Biotin ligase Bira, and biotinylation performed in vivo. Equal amounts of these cells and heavy SILAC control cells were mixed and incubated with streptavidin beads. Biotinylated proteins were recovered with four methods using on bead digestion in combination with off-gel fractionation (2 different protocols), digestion prior to biotin capture and elution from the beads followed by in gel digestion. Samples were analyzed using LC-MS/MS.

Results
Using SILAC labeled unbiotinylated HEK cells we determined the variability of background proteins and defined a cut off for distinguishing between truly enriched biotinylated proteins and unspecific background. Among the protocols evaluated, on bead digestion in combination with off gel fractionation resulted for both experiments in identification of ~3000 proteins containing 75% and 72% biotinylated proteins, respectively. Incubation of peptides on beads resulted in the identification of ~100 proteins out of which 17% were biotinylated. Elution of biotinylated proteins followed by in gel digestion identified ~700 proteins with 75% being biotinylated.

Conclusions
We observed similar enrichment capacities for all methods except enrichment of pre-digested peptides. On bead digestion in combination with offgel fractionation resulted in highest numbers of identified proteins as well as the best enrichment efficiency.

Keywords
Biotin, protein enrichment, on bead digestion

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Quantitative Proteomic Analysis of the Interactome of mammalian S/MAR (scaffold/matrix attachment region) elements

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Background
Matrix Attachment Regions (MARs) are eukaryotic regulatory DNA sequences, believed to spatially organize chromatin within the nucleus by mediating binding of DNA to the nuclear matrix. Upon chromatin integration they exhibit potent gene anti-silencing and expression augmentation activities. Due to these properties, MARs found many biotechnological applications, also in stably replicating episomal vectors that without integrating into host DNA still provide (MAR-dependent) long-term transgene expression. Despite the well-documented function of MARs, their precise mode of action is still enigmatic, a fact that can mainly be attributed to our limited knowledge concerning MAR-binding proteins.

Methods
To address this question we apply reverse ChIP, a well-established, SILAC-based proteomics method for DNA-protein interaction screening. Functional MARs are immobilized on magnetic beads and used for pull-down assays employing HeLa nuclear extract and solubilized chromatin fractions. Enriched proteins are identified and quantified by nanoLC-MS/MS. For bait design we focus on human interferon ß-gene MAR, which harbors a base pair unwinding region (BUR) deemed to be essential for MAR function; as a control we utilize MARs bearing mutated BUR or reversed WT sequences.

Results
Performing experiments in HeLa nuclear extract samples, we detect enrichment of 17 and 22 proteins with reverse WT and BUR mutant control, respectively. Half of those proteins are involved in gene expression. Interestingly, we notice specific enrichment of RNA splicing factors in case mutant control is applied. Using solubilized chromatin fraction a similar scenario with enrichment of altogether 35 proteins on WT MAR is observed. Likewise, about 80% of candidates identified with BUR mutant control are again proteins related to RNA splicing.

Conclusions
Using reverse ChIP we succeeded in identifying a number of potentially novel MAR binders. Selection of different control sequences helped to link candidates binding specificity directly to BUR-dependent and independent MAR binders.

Keywords:
MAR DNA-interactome, Reverse ChIP, SILAC

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ImportOmics: A New Method for Charting Organellar Proteomes by Quantitative Mass Spectrometry

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The majority of proteins in eukaryotes are nucleus-encoded and synthesized in the cytosol. To reach their final destination, they need to follow different import pathways into various organelles. The resulting protein inventories define the molecular identity and specific functions of different organelles. However, methodology to examine whether a protein is transported into an organelle is largely limited to the study of single proteins. This renders current knowledge about imported proteins incomplete, limiting our understanding of the biogenesis and functions of organelles.

We use inducible RNAi-mediated knockdown of essential organellar import factors to impair protein import combined with quantitative high-resolution mass-spectrometry and bioinformatics data analysis to globally chart an organelle's importome.

We demonstrate the high potential of our new ImportOmics method by targeting the archaic translocase of the mitochondrial outer membrane (ATOM), the main entry gate into mitochondria in the single-cell parasite Trypanosoma brucei. RNAi-mediated knockdown of its central component ATOM40 allowed us to define the mitochondrial importome of T. brucei comprising 1,120 proteins with more than 300 novel mitochondrial constituents. Importantly, ImportOmics allows for the exact localization of proteins with dual or multiple locations. To highlight ImportOmics' specificity and versatility, we targeted import factors of different organellar protein import pathways like the sorting and assembly machinery for import of proteins into the mitochondrial outer membrane, the mitochondrial intermembrane space (IMS) assembly pathway mediating import into the IMS and glycosomal import pores. As a result, we report 25 new IMS and 26 new glycosomal proteins.

ImportOmics allows for globally studying organellar proteomes and in vivo protein import into organelles. It can be adapted to different protein import systems by targeting a distinct, central component of the protein import machinery and can be used in other organisms in which inducible knockdown of the target component is feasible.

SILAC, RNAi-mediated-knockdown, proteomics, protein-import, bioinformatics

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Quantitation of putative colorectal cancer biomarker candidates in serum extracellular vesicles by targeted proteomics

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Background: Colorectal cancer (CRC) is one of the major cancer related death in the world. Although, the numerous research elucidate the mechanism of CRC occurrence and development, the effective biomarker and therapy have not yet been established. Recent advances in target proteomics have enabled high-throughput verification of hundreds of biomarker candidate proteins. Within the past few years, extracellular vesicles (EVs) have emerged as important mediators of intracellular communication and to play the pathologic role in several disease including cancer. In this study, we aimed to verify previously reported CRC biomarker candidate proteins in EVs from CRC patient serum by a targeted proteomic method called selected reaction monitoring (SRM).
Methods: PubMed literature search from 2003 to 2014 and our recent report represent 723 CRC biomarker candidate proteins that were functionally correlated with CRC. First, we performed a shotgun proteomic analysis to search for the biomarker candidates and were able to detect 356 proteins in serum EVs. Then, we quantitated these target proteins by SRM using serum EVs of healthy control (n=26) and CRC patients with (n=25) or without (n=26) metastasis.
Results: 37 peptides of 22 proteins showed significant difference between three groups, and these sensitivity were higher than conventional marker CEA.
Conclusions: These result suggest that target proteomics was effective for biomarker discovery, and identified peptides were potential biomarker of CRC.
Keywords:Colorectal cancer, Biomarker, Extracellular vesicles, Selected reaction monitoring

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MaxQuant Top-Down: A multifunctional software package for quantitative top-down proteomics

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Background
Top-down mass spectrometry-based proteomics allows the study of intact proteins on the proteoform level. One challenge of this technique is the automated analysis of high-resolution mass spectra, which are highly complex and therefore require advanced algorithms in order to be interpreted properly. Even though there exist many algorithms and implementations, the situation of software analysis remains unsatisfactory because the algorithms are mostly not integrated into free available multifunctional software packages and the automated identification and quantitation of proteoforms is still challenging.

Methods
Based on MaxQuant for shotgun-proteomics the top-down version takes advantage of existing technology and offers additional functionalities that are needed to process high-resolution top-down mass spectra. To enable the automated spectral deconvolution of complex top-down mass spectra we implemented several algorithms, which can determine of the masses and charge states of proteoforms with overlapping isotope patterns. The powerful viewer part allows the user to visually inspect and validate the deconvolution results by comparing the mass spectra with their theoretically predicted counterparts. The results of the analysis can be exported and statistically evaluated with the Perseus software platform.

Results
Using mass-spectrometry data of proteins with masses around 20kDa and 150kDa, respectively, we demonstrate the power of the implemented deconvolution algorithms in terms of preciseness, robustness, and speed. Furthermore, we give an overview over the graphical user interface and the general usage of the software package.

Conclusions
MaxQuant Top-Down pursues the goal to provide all tools for the complete analysis of top-down mass spectra within one user-friendly software package. This first version includes different deconvolution algorithms and a graphical user interface to perform the analysis and inspect its results. In future versions the software will be extended by modules for identification and quantitation of proteoforms.

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Determination of the RNA / protein ratio in human cells

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Background: The correlation between mRNA and protein levels has been debated for long. It is an issue of paramount importance to address the conflicting reports for both proteomics and genomics fields. If RNA could be used as proxies for protein levels, the value of transcriptomics data performed so far in cell lines and (tumor) tissues would increase significantly and gain an unprecedented preclinical and clinical value. We set out a complementary proteomics and genomics platform to address this fundamental question in biology.

Methods: We generated RNA-seq data and performed (shotgun) proteomics analyses using a TMT10plex approach in 9 different cell lines. The lysates were normalized to a cell pool that was absolutely quantified using the PrESTs technology. MS3 quantification was performed using an Orbitrap Fusion instrument. Both proteomics and genomics analyses were performed in three biological replicates.

Results: We identified ~7000 proteins and we determined the RNA/Protein (RTP) ratio and the absolute copy number for 5-6000 entries across the employed cell lines. Overall, our data show a very high correlation (R=0.9).

Conclusions: RNA data can be used to predict protein expression levels. This paradigm holds at the steady state.

Keywords: TMT; MS3; RNA-seq; RTP; PrESTs

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Triple extraction enables mass spectrometry-based proteomics and phospho-proteomics and is compatible with robust multi-omics analysis

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Omic strategies have become essential tools in the investigation of cellular and tissue molecular characterization nowadays, proving crucial in deciphering the biology of a wide range of diseases, namely cancer. The integration and correlation of different large-scale approaches represents a major challenge for researchers, that have to deal with intra-tumor heterogeneity to select comparable sections to analyse through different techniques. Triple extraction (TE) has already been implemented for obtaining DNA, RNA and proteins from the same tissue section, thus minimizing errors due to selecting heterogeneous areas within the tumour. Large-scale genomics and transcriptomics have been successfully performed in TE-obtained material. Nevertheless, TE has not been tested for its compatibility with subsequent gel-free mass spectrometry-based analysis of proteins. Here, we demonstrate that TE samples might be analysed through mass spectrometry and, importantly, that not only the proteins but also the phosphorylated proteome is well conserved.
Scaber cells were harvested either in urea buffer or through TE. Proteins were subjected to in-solution digestion, SAX peptide fractionation and enrichment of phosphorylated peptides using TiO_2 procedure, followed by analysis in an Orbitrap Fusion.
Proteins extracted through the different methods were essentially involved in the same biological processes and located in equal cellular compartments, demonstrating no bias of the TE for any particular pool of proteins. We detected almost 18000 phosphorylated sites in our analysis (enrichment efficiency >92%). Notably, the total amount of identified sites was comparable in both techniques, with even slightly higher numbers in TE. Class I sites (loc prob >0.7) were equally detected in urea or TE-obtained samples and distribution of sites (S, T, Y) was comparable between both techniques. We detected 901 novel sites in our dataset.
In conclusion, TE represents an attractive strategy to combine different omic techniques on essentially the same tissue sample, compatible with mass spectrometry-based proteomics.

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Automated, versatile and efficient sample clean-up using HILIC-functionalised magnetic microparticles

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Background
In-depth proteome coverage is achieved through the addition of detergents and chaotropes to efficiently disrupt cells and solubilize proteins. Buffers containing components including preservatives, salts, reducing and alkylating agents are added in order to keep proteins in suspension and to achieve complete enzymatic cleavage, a critical step in bottom-up MS proteomics workflows. These components are however not compatible with MS analysis resulting in ion-suppression and system contamination. We outline a novel HILIC-based method for sample clean-up using mixed-mode HILIC magnetic microparticles, allowing for removal of a range of contaminants including detergents, chaotropes and salts, in a versatile automatable format.

Methods
Cell extracts were solubilized using detergents and chaotropes and cleaned-up by binding to MagReSyn® HILIC, followed by 4hr on-bead tryptic digestion and subsequent MS analysis using an AB Sciex TripleTOF 6600 coupled to Dionex nanoRSLC. The workflow was automated for HT sample preparation using a magnetic bead handling station. Spectral data was analysed using Peaks Studio 6 (Bioinformatics Solutions Inc) with Swiss-Prot mammalian database supplemented with common contaminating proteins. A 0.1% and 1% FDR cut-off was applied at the PSM and protein levels respectively.

Results
The HILIC-based clean-up method was able to efficiently remove a range of MS incompatible contaminants used during protein extraction, solubilisation and digestion, including SDS, CHAPS, NP-40, Urea, PBS, Tris, DTT and IAA. The integration of all steps into a single automated workflow allowed for processing of 12 (KingFisher™ Duo) or 96 (KingFisher™ Flex) samples in parallel in under 5hrs (25 or3 min per sample). The automated method showed high technical reproducibility.

Conclusions
Efficient and rapid protein clean-up and digestion was achieved from a range of common contaminants using an automatable HILIC magnetic bead based workflow, providing reproducible high-throughput processing of up to 96 samples in parallel.

Key words
Proteomics, clean-up, high-throughput, HILIC, magnetic

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'Omics' exploration of the human tears

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Background:
Tears represent an exciting promising fluid for biomarkers discovery. Indeed, their complex molecular composition (proteins, lipids, metabolites, nucleotides) is highly regulated depending on several factors including ocular and systemic diseases. Furthermore, their easy way to collect, invasiveness, timeless and absence of side effects make them particularly attractive for clinical uses. Nevertheless, to date, this fluid is still poorly studied. Here we present an integrative analysis of the human tear composition using both proteomics and metabolomics approaches.
Methods:
Tears of healthy subjects were collected with Schirmer's papers then pooled. For the proteomics part, trypsin liquid digestion and off-gel electrophoresis (OGE) fractionation were performed, then analyses were done using Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ (Thermo Fisher Scientific) coupled to a liquid chromatography. Resulting files were searched against the SwissProt database (version 2016, Homo sapiens taxonomy) with a false discovery rate of 1% at both protein and peptide levels. Metabolomics analysis was achieved using a large scale LC-MRM/MS approach, on a hybrid triple quadrupole-linear ion trap QqQLIT coupled to liquid chromatography. Pick integration was done using Multiquant (Sciex).
Results:
Globally 3309 and 2021 proteins were identified with 1 and 2 unique peptides respectively after excluding keratins. Pathway analyses highlighted among others the glycolysis and the coagulation and complement cascades. Furthermore the metabolomics analysis permitted to identify up to 195 metabolites involved in interesting pathways such as alanine, aspartate and glutamate metabolism, pentose phosphate pathway and citrate cycle (TCA cycle). These two last pathways, which are part of the glycolysis, were completely related to the proteomics results.
Conclusion:
This unconventional analysis of tears by exploring both proteome and metabolome from healthy subjects drastically improved the knowledges of this fluid. Using two complementary types of data strengthened our results and brought information about how to combine 'omics' data.
Keywords: tears, proteome, metabolome, integrative pathways

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In situ characterization of infiltrated immune cells of murine EAE by MALDI imaging mass spectrometry

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Background

Experimental autoimmune encephalomyelitis (EAE) is the most common experimental model for multiple sclerosis (MS). Previously, we have confirmed that imaging mass spectrometry (IMS) is well suited for the visualization of spatial distributions of infiltrated cells population in EAE brains and spinal cords. Here we have tried to validate and compare IMS data to antibody based immunohistochemistry (IHC) for candidate peptides or proteins.

Methods

Brains and spinal cords from murine EAE were resected and snap-frozen in liquid nitrogen. Ten µm frozen sections were cut and transferred to Indium tin oxide (ITO) coated glass slides. The sinapinic acid were sprayed as a matrix. IMS data were acquired on a MALDI TOF/TOF mass spectrometer ultrafleXtream (Bruker Daltonics) with a spatial resolution of 50-80 µm in linear mode and the ions were detected in a mass range of m/z 2,500 to 25,000. Visualization and statistical analysis were done using FlexImaging and SciLS Lab 2016a.

Results

The infiltrated immune cells were observed at hippocampus and cerebellum as well as in spinal cords of EAE from preclinical to clinical stages. These cell clusters were easy to be found with HE staining and were collected with laser microdissection (LMD). We have identified several marker proteins directly from LMD samples. For these cell lysates, we have identified distribution of the proteins which is perturbed with EAE pathogenesis by IMS.

Conclusion

The current study can help to define the neuroinflammatory process of the EAE with a single to several proteomic marker. This strategy can be applied to human neuropathological study and opens the way for neuro-inflammation-related proteomic biomarker research.

Keywords

Multiple sclerosis (MS), Experimental autoimmune encephalomyelitis (EAE), Imaging mass spectrometry (IMS), Neuroinflammation

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Optimisation of methodologies for the in-solution preparation of human Synovial Fluid prior to SWATH-MS analysis.

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Background:
Synovial fluid is of clinical significance used in the diagnosis of several arthritic conditions. Generally, plasma samples are cleaned-up or depleted before mass spectrometry analysis as 12 highly abundant proteins are responsible for 90% of the total protein content. Synovial fluid does not contain all 12 of these proteins at high abundance, but does contain the same two most abundant blood proteins (albumin and immunoglobin; 32% & 14.5% total synovial fluid protein content respectively). Therefore, there may be a need to deplete the TOP2 plasma proteins from synovial fluid in order to unmask biomarkers expressed at lower levels in the fluid.

Methods:
Synovial fluid samples were prepared with/without immunodepletion using TOP2 immunodepletion spin columns. These columns have been developed to work with max. 10µl serum or plasma. Synovial fluid contains a lower concentration of albumin (8g/l vs ~55g/L), so a range of higher volumes of synovial fluid were loaded onto the column to identify the saturation point of the column's binding capacity.
BCA assays were used to determine flow through protein concentrations and samples were run on an SDS-PAGE gel with Coomassie Blue staining for visualisation. Samples then underwent in-gel digestion and compared with DDA-MS runs both for characterisation of the synovial fluid sample preparation protocol and to produce a spectral library specific to synovial fluid for use in SWATH-MS experiments.

Results:
Up to 30µl of synovial fluid can be loaded on a Top2 depletion column before saturation. Approximately 350 proteins were identified with the DDA runs including several which have been described as present in synovial fluid previously.

Conclusions:
These techniques will provide a general understanding of the protein composition of synovial fluid and will form the basis of an in-solution sample processing methodology for SWATH-MS for potential new biomarkers.

Keywords:
Immunodepletion, Synovial fluid, Spectral library, SWATH-MS

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LC-SRM targeted quantification of 20S proteasome complexes stoichiometry and dynamics using absolute SILAC

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Background: The proteasome is involved in the degradation of most intracellular proteins. The 20S core particle is composed of 14 different subunits organized in barrel-like structure of four stacked rings (α7β7β7α7). Four subtypes of 20S complexes containing either standard catalytic subunits (β1, β2, β5), immunosubunits (β1i, β2i, β5i), or a mix of both subunit types, have been observed in most human cell types(1). Other particular 20S subtypes are more tissue-specific like the thymoproteasome and the spermatoproteasome(2), which precise subunit compositions have never been described. A multiplexed method for the absolute quantification of all 20S proteasome subunits in a one shot assay has thus been developed to determine the composition and the subunit stoichiometry of human 20S complexes.

Methods: We combined SILAC labeling and a multiplexed LC-SRM mass spectrometry method to determine the absolute concentration of all 20S proteasome subunits. We could then obtain the exact stoichiometry of various 20S proteasome subtypes present in cell lines and tissues of broad origins.

Results: The LC-SRM method we developed was first optimized and validated by comparison to an existing Elisa reference assay. Its superiority over a label-free MS1-based quantification(3) was also demonstrated. Then, it has been used to accurately quantify the tissue-dependent stoichiometry of 20S proteasome subtypes as well as to monitor the dynamics of 20S proteasome composition in response to IFNγ or during spermatogenesis.

Conclusions: The absolute concentration and exact stoichiometry of 20S proteasome could be determined with high precision(>92%), accuracy(>90%), and sensitivity (<1 fmol on column) in various biological samples. This is the first study reporting the determination of macromolecular complexes stoichiometry based on the isotopic dilution of heavy-labeled proteins. Knowledge of proteasome composition could help for their selective inhibition in personalized therapies.

Keywords: Proteasome; Targeted proteomics; stoichiometry; macromolecular complexes; absolute quantification

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Analysis of a super-sensitive in vitro diagnostic platform for Troponin I, a cardiovascular biomarker

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Background

Ischaemic heart disease is the leading cause of death worldwide and has a considerable impact on quality of life. Cardiac troponin (cTn) is the reference standard biomarker for AMI. With standard cTn assays, serial blood sampling was required over 6-12 hours in order to detect increasing concentrations of cTn. With high sensitivity assays, this can be achieved with samples drawn 3-4 hours apart. The assays have improved sensitivity to detect low quantities and subtle changes in troponin. This has implications for the future of manging heart disease such as determining the risk of post-AMI heart failure, patient prognosis and identifying those with a higher chance of heart disease in the general population. This improved sensitivity also has the benefit of detecting troponin in healthy individuals, which could 'rule out' AMI with a single blood test.

Methods

The Singulex Clarity is an automated Single Molecule Counting immunodiagnostic platform that allows precise measurement of troponin down to levels undetectable through previous approaches. We conducted a blinded retrospective study on a cohort of samples collected from patients whom presented at the Central Manchester Emergency Department (ED) with chest pains. These patients had samples taken at two time points; upon admittance to the ED and 3 hours after admittance. Lithium heparin plasma was run on the Clarity to analyse troponin levels.

Results

Troponin was detected in all samples tested with a dynamic range of expression of 0.23pg/ml-6,700pg/ml. The troponin I measurements from the Clarity system were compared to the outcome measures obtained for the cohort; results will be discussed.

Conclusion

The aim was to evaluate if super sensitive assays such as the Singulex Clarity can rapidly rule out acute coronary syndrome, avoiding unnecessary hospital admissions and saving NHS money.

Keywords

Clinical biomarkers, Troponin I, cardiovascular disease, in vitro diagnostics, myocardial infarctions

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Unraveling amyloid beta-pathology in Alzheimer's Disease with MALDI Imaging Mass Spectrometry

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Background Neuropathology of Alzheimer’s disease (AD) is characterized by the accumulation and aggregation of Amyloid β (Aβ) peptides into extracellular plaques of the brain. The Aβ peptides are generated from amyloid precursor proteins (APP) by β- and γ-secretases. While a variety of Aβ peptides were identified, detailed production and distribution of individual Aβ peptides in pathological tissues of AD is not fully addressed. Here, we adopt MALDI-imaging mass spectrometry (MALDI-IMS) on autopsied brain tissues to obtain a comprehensive protein mapping.
Methods Human cortical specimens for IMS and immunohistochemistry (IHC) were obtained from brains at the Brain bank at Tokyo Metropolitan Institute of Gerontology. For all brains registered at the brain bank, we obtained written informed consents for their use for medical research from the patients or their families. This study was approved by the ethics committee at each hospital or institute. 10 µm cryosections were cut and transferred to Indium-Tin-Oxide (ITO) coated glass slides. Spectra were acquired using the rapifleX MALDI Tissuetyper in positive linear mode in a mass range of m/z 2,000 to 20,000. Sinapinic acid was uniformly deposited on the slide using the ImagePrep device. Visualization and statistical analysis were used FlexImaging and SciLS Lab 2016a.
Results The current analysis clarifies that Aβ1-42 and Aβ1-43 were selectively deposited to senile plaque and shorter Aβ peptides were deposited to leptomeningeal blood vessels and parenchymal arterioles of brains. Distinct depositions of N-terminal truncated Aβ40 and Aβ42 including pyroglutamylation (pE) at Glu-3 were also visualized. Aβ1-41 was detected both with MALDI-IMS and IHC suggesting that a single amino acid alteration at the C-terminus of Aβ results in drastic distribution changes.
Conclusions MALDI-IMS in future, with the further development of the analytical platform, would replace conventional IHC using specific antibodies.
Keywords Imaging Mass Spectrometry, Alzheimer’s Disease, Senile Plaque, Cerebral Amyloid Angiopathy

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A comprehensive HLA peptidomic resource used to generate immunotherapeutics based on high affinity T-cell receptors

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Background
Peptides presented to the immune system on HLA complexes are valuable targets for immunotherapeutic treatments of cancer, viral infection and autoimmune disease. Analysis of HLA peptides by mass spectrometry has expanded considerably in recent years and represents the most reliable method for HLA-peptide identification. Identifying the full complement of peptides that derive from a protein and are presented on a major HLA restriction is a vital step in generating commercially viable products.
Method
Our approach to understanding the targetable human HLA peptidome is based on three key principles; achieving full proteome coverage, maximising individual protein coverage, and focusing on dominant HLA restrictions. By integrating novel cell biology, mass spectrometry, and bioinformatic technologies we have dramatically increased the consistency and depth of the HLA ligandome captured.
Results
Our current data management system contains HLA peptidomic data from over 900 experiments, including hundreds of distinct cell line models spanning 30 distinct tissue indications. All lines contain at least one major HLA-allele restriction, with high bias toward the restriction HLA-A*02:01. The natural diversity in gene expression gained by using this dataset has allowed us to achieve near total coverage of the proteome, with 90% of the proteome captured for HLA-A*02:01.
Identified peptides are fed a T-cell cloning facility to generate peptide-HLA specific T-cells. TCRs isolated from these cells are then engineered into soluble molecules (mTCRs). Our immune-activating therapeutics (ImmTACTM) are created by coupling high-affinity mTCRs with an anti-CD3 scFv domain. Anti-CD3 mediated T-cell redirection is a clinically-validated, highly potent therapeutic mechanism of action. ImmTAC molecules activate polyclonal T cell responses targeted toward cells presenting the appropriate cancer-associated antigen.
Conclusions
An integrative approach to HLA peptidomics has delivered a powerful reference database of HLA peptides that is the source for generating potent soluble immunotherapeutics
Keywords
Human-Leukocyte Antigen; T-cell receptor, Mass spectrometry, Ligandome, Immunotherapy

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Discovery of the proteomic profile to predict the renal function decline in kidney disease

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BACKGROUND

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic disorder, characterized by development and enlargement of cysts in the kidneys and other organs. Loss of glomerular filtration rate is a late stage marker of disease, but novel therapies are focusing on reversing earlier stages of the disease which is an area of unmet clinical need.

The work here described aimed to define serum proteomic patterns in ADPKD patients, identifying protein profiles associated with more rapid loss of glomerular filtration rate (GFR) and disease progression.

METHODS

Serum samples from chronic kidney disease in stages 2-3 ADPKD with known GFR trajectory were selected. Patients were categorised in rapid or standard GFR loss with at least 4-years follow-up data. Samples were pre-processed and digested with trypsin prior to SWATH-MS data acquisition on a SCIEX Triple TOF-6600 with 100 variable windows. MS Data was processed to determine the top 10% deferentially expressed proteins between rapid and standard progression, and analysed using STRING and GO analysis.

RESULTS

Data from STRING analysis suggested that patients from the rapid GFR loss group had statistically significant pathway interconnectivity. Three of the proteins identified (BHMT, GOT1 and LDHB) were connected to the pathway of cysteine and methionine metabolism. These pathways have been previously implicated as important in renal failure. These samples also showed high levels of super-oxide dismutase in rapid GFR loss and high levels of vascular adhesion protein which have previously been described as potential markers of chronic kidney disease.

CONCLUSIONS

The results demonstrate that a global proteomics approach is able to provide mechanistic insight into progression of a little understood chronic disease. Several proteins have been identified which could potentially be used as clinical biomarkers for patients with a poor prognosis who require more aggressive therapeutic interventions.

KEYWORDS

Chronic kidney disease, prognostic biomarkers, SWATH-MS

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Phosphoproteomic analysis of the neural progenitor to oligodendrocyte progenitor cell differentiation

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Background:
Oligodendrocytes are the myelinating cells of the central nervous system. They originate from neural stem cells through a two-step differentiation mechanism. First multipotent neural progenitor cells differentiate to oligodendrocyte precursor cells and then these cells further differentiate into mature oligodendrocytes. These differentiation processes are only incompletely understood. We investigated the first differentiation step using free floating spheres of neural progenitor cells called neurospheres. In a previous study, we identified several regulated kinases during this process. We therefore, performed a large scale phosphoproteomics experiment to study changes of the phosphoproteome during the differentiation process.

Methods
Neurospheres were differentiated into oligospheres using B104 conditioned medium and samples were taken at an early, medium and late time points of the differentiation process. We used 3 plex dimethyl labeling and separated combined samples to 12 fractions by strong cation exchange (SCX) chromatography. Fractions were phosphopeptide enriched by TiO2 method. Phosphopeptide enriched and flow through fractions analyzed by LC-MS/MS. The whole experiment was performed in 3 biological replicates.

Results:
We identified 10680 unique phosphopeptides covering 9053 phosphorylation sites. Of the 6667 class 1 phosphorylation sites identified, 3732 were already in the PhosphoSitePlus database. With 2935 class 1 phosphorylation sites, 44% of the identified sites were not yet available in the PhosphoSitePlus database representing potentially novel phosphorylation sites. Proteins with phosphorylation sites that were up- or downregulated 2-fold or more were subjected to a GO enrichment analysis.

Conclusions:
The results of the GO enrichment show enrichments with above nominal significance in GO categories that are expected to be affected in such a differentiation process. Especially proteins in the 'Glial cell differentiation' category potentially play important roles in the differentiation such as NGFI-A-binding protein 2 (Nab2) and Rap1 GTPase-activating protein 1 (Rap1gap).

Keywords:
Phosphoproteomic, Neurospheres differentiation, TiO2 , LC-MS/MS

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Evaluation of KRASG12C cysteine-reactive inhibitors by Proteomic Cysteine Profiling in living cells

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Background
RAS is one of the most crucial anti-cancer target, as oncogenic mutations in RAS are found in 30% of human cancers. The KRAS mutation has long been considered "undruggable", but continued efforts and new approaches generated renewed interest in the development of inhibitors. Here, we implemented a proteomic cysteine profiling approach to assist the development of new KRASG12C inhibitors by assessing their efficiency to target KRASG12C and their selectivity across free cysteine residues.

Methods
NCI-H358 cells were treated with the KRASG12C inhibitor ARS-853 used as a reference or two early chemical series of KRASG12C inhibitors. After lysis, surface exposed cysteine residues were labeled with desthiobiotine iodoacetamide. Proteins were digested and desthiobiotinylated peptides enriched using streptavidin agarose beads and analyzed by nanoLC-MS/MS.

Results
The quantitative analysis of ARS-853 treated NCI-H358 cells led to the quantification of 7,500 desthiobiotinylated cysteine residues, from 3,200 proteins. Among them, five cysteine residues were statistically identified as displaying significant engagement. Only KRASG12C was affected in a dose-dependent manner, therefore representing the most potently modified target. The same workflow was also applied to NCI-H358 cells treated with the two series of KRASG12C inhibitors. The results obtained allowed us to evaluate them first in terms of efficiency by comparing KRASG12C engagement obtained with the different molecules, secondly in terms of selectivity by comparing the number of off-targets identified.

Conclusions
Proteomic cysteine profiling is a powerful method to assess the efficiency and the covalent selectivity of KRASG12C inhibitors, or of any small molecules targeting reactive cysteine residues. Therefore, it represents a very helpful method for assisting the development of improved reactive cysteine residue inhibitors.

Keywords
Proteomic Cysteine Profiling, KRASG12C, Cysteine-Reactive Inhibitor, Drug development, Drug Selectivity

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Novel proteomic signatures of human blood vessel maturation revealed by SWATH analysis.

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Background
Vasculogenesis is the mechanism by which new blood vessels are formed. It has an impact on numerous physiological processes, such as appropriate organ development, and is implicated in various pathologies, e.g. tumourogenesis. Yet, the molecular changes underlying human blood vessel formation and maturation remain unresolved. The placenta is an organ forming de novo over a period of several months of pregnancy to support fetal growth, offering an unique model to observe aspects of human blood vessels in a natural, non-pathological setting. Therefore, we have performed proteomic analysis of human placental arteries at different stages of development to investigate the protein changes underlying human blood vessel maturation.
Methods
Following written informed consent (LREC 10/H0906/71), human placenta biopsies were obtained from 1st (7-12 weeks, n=9) and 3rd (39-40 weeks, n=7) trimesters of normal pregnancy. Chorionic plate arteries were isolated, homogenized, trypsin digested and analysed with LC-MS/MS (U3000 RSLCnano/TripleTOF6600) using SWATH acquisition. Proteins were quantified in PeakView version 2.2, using Pan-Human spectral library and differential abundance was determined with a modified t-test, followed by pathway analysis.
Results
3586 distinct proteins were quantified, with 1073 proteins differing significantly between the 1st and the 3rd trimester (FDR 0.01 and >1.5-fold change). 482 proteins were up- and 591 were down-regulated at term. Notable changes involved: (i) down-regulation of proteins involved in RNA splicing (e.g. 33 spliceosome-related proteins) and translation (63 ribosomal proteins); (ii) up-regulation of those involved in ECM/myofilament/cytoskeletal integrity; (iii) up-regulation of the Serpin superfamily (12 of 17 identified) indicative of possible regulation of matrix remodelling and endocrine/immune signalling.
Conclusions
Our study reveals an enormously dynamic proteomic milieu associated with human blood vessel maturation and provides a comprehensive characterization of the major underlying protein abundance changes. We identify key processes/molecules involved and provide a rich resource for subsequent, more focused experimentation.

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Two-dimensional thermal proteome profiling in living cells, cell extracts and beyond

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Background:
With the Cellular Thermal Shift Assay (CETSA) introduced by the group of Paer Nordlund (Molina et al. Science 2013) it is possible to monitor drug-protein interactions in living cell. We demonstrated that the combination of CETSA with quantitative mass spectrometry and a dedicated bioinformatic analysis platform enables the determination of thermal stability data on a proteome-wide scale (Savitski et al. Science 2014). This approach, also termed Thermal Proteome Profiling (TPP) enables the hypothesis-free identification of drug targets and off-targets. We refined TPP by combining temperature dependent and concentration dependent experiments into one experimental scheme (2D-TPP) to allow the comprehensive determination of drug target affinities across the proteome.

Results:
Using the histone deacetylase inhibitor panobinostat as a model system we compared how experimental parameters in the 2D-TPP setup influence the ability to identify it's targets and off-targets and to distinguish changes in thermal stability caused by direct target binding from indirect effects. Experiments performed in live HEPG2 cells were compared to three different cell extract protocols. We found that membrane proteins were underrepresented if cell extracts were generated without addition of detergents. Better coverage of membrane proteins was achieved when the detergent was either added prior to or after the heat treatment. The latter can be applied to 'crude' cell extracts where cells were opened by shear force but not cleared for insoluble particles.
Whilst heating of detergent treated cell extracts led to altered melting points of target proteins and incomplete coverage of panobinostat targets, detergent solubilization of heat-treated extracts enabled similar coverage as live cell treatment and did not substantially alter thermal stability of target proteins.

Conclusions:
A new protocol for 2D-TPP with cell extracts will enable the detection of the drug effects on their targets/off- target extracted from primary material allowing to measure personalized target engagement.

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Combined biomarker panel shows improved sensitivity for the early detection of ovarian cancer

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Background:
Ovarian cancer has the highest mortality rate of all the gynaecological cancers with an estimated 4,128 deaths in the UK in 2014. This is due to its typically late diagnosis, with 5 year survival rates of 5% in those diagnosed at Stage IV. If diagnosed early, at stage I, the 5 year survival rate rises to >90%. There is an unmet need for tests that enable earlier diagnosis, which would increase survival.

Methods:
This nested case control study used samples from the UKCTOCS trial. The sample set consisted of ~500 serum samples from 49 OC subjects and 31 controls, with serial samples spanning up to seven years pre-diagnosis. The set was divided into: (i) a discovery set which included all women with only two samples from each woman, the first at < 14 months and the second at > 32 months to diagnosis; and (ii) a verification set which included all the serial samples from the same women spanning the 7 year period. A previous study by the authors' detailed the identification of multiple putative biomarkers using high-through-put quantitative proteomics. The performance of a number of these markers to detect cancers, pre-diagnosis, was assessed.

Results:
Biomarker panels outperformed CA125 alone for the earlier detection of ovarian cancer. These panels were able to identify the most aggressive cancers, in addition to providing an increased lead time for Type I and Type II OC compared to CA125.

Conclusions:
The combined biomarker panels, identified by high-through-put mass spectrometry, outperformed CA125 up to 3 years pre-diagnosis. It identified cancers missed by CA125, providing increased diagnostic lead times for Type I and Type II OC, identifying the more aggressive Type II cancers. Indicating that these markers can improve on the sensitivity of CA125 alone for the early detection of ovarian cancer.

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PHOSPHOPROTEOMIC PROFILING OF HEART TISSUE: FROM MOUSE MODEL TO PATIENT SAMPLES

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BACKGROUND: Heart failure (HF), a pernicious disorder resulting from a variety of cardiovascular diseases (CVDs), is now the leading cause of mortality in North America. Perturbations in signaling pathways associated with late stage CVD/HF events under different pathological conditions have been identified, but the critical early regulatory perturbations that precede and ultimately result in functional impairment of the heart are not yet known. Differential phosphorylation has been widely implicated as a central regulatory and signaling mechanism in a variety of cellular processes associated with the initiation and progression of CVDs, but few systematic global surveys have yet been reported.
METHODS: We took advantage of recent advances in quantitative mass spectrometry (MS)-based techniques to perform large-scale identification and quantification of phosphorylation sites in different cardiac pathologies. This involved: optimized sample preparation and protein extraction protocols, chromatographic separation and enrichment of phosphopeptides, refined data searching and processing, and bioinformatic systems biology-based extraction of meaningful information from the acquired data.
RESULTS: Here, we report the development of an effective proteome-scale quantitative phosphoproteomic pipeline compatible with the use of label-free and isobaric tag-based quantification approaches and its application to the analysis of signaling pathway changes in a mouse model of dilated cardiomyopathy (DCM) and human heart tissue from patients suffering from several different cardiac pathologies. This resulted in the identification and quantification of 7,589 unique putative phosphorylation sites on 1848 mouse cardiac phosphoproteins, and in excess of 6500 phosphorylation sites on more than 1600 human cardiac proteins (up to the date of abstract submission with ongoing sample profiling).
CONCLUSIONS: This unprecedented profiling allowed for the bioinformatic/systems biology-based elucidation of hundreds of dysregulated signaling pathways and other biological process in cardiac pathology, many of which have not been reported before including aberrant Notch-1 signaling in dilated cardiomyopathy.

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Optimization of quantitative proteomic analysis of clots generated from plasma of patients with venous thromboembolism

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Background
It is well known that fibrin network binds a large variety of proteins, including inhibitors and activators of fibrinolysis, which may affect clot properties, like stability and susceptibility to fibrinolysis. Specific plasma clot composition differs between persons and may change in disease states. However, so far the plasma clot proteome has not been in-depth analyzed, mainly due to technical difficulty related to the presence of a highly abundant protein – fibrinogen and fibrin that forms a plasma clot.

Methods
The aim of our study was to optimize quantitative proteomic analysis of fibrin clots prepared ex vivo from citrated plasma of the peripheral blood drawn from patients with prior venous thromboembolism. We used a multiple enzyme digestion filter aided sample preparation (MED FASP) method combined with LC-MS/MS analysis performed on a Proxeon Easy-nLC System coupled to Q Exactive HF mass spectrometer. We also evaluated the impact of peptide fractionation with pipet-tip strong anion exchange (SAX) method on the obtained results.

Results
Our proteomic approach revealed >500 proteins repeatedly identified in the plasma fibrin clots from patients with venous thromboembolism. The multienzyme digestion (MED) FASP method using three different enzymes: LysC, trypsin and chymotrypsin increased the number of identified peptides and proteins and their sequence coverage as compared to a single and double step digestion. Peptide fractionation with SAX protocol slightly increased the depth of proteomic analyses, but also extended the time needed for sample analysis with LC-MS/MS.

Conclusions
The MED FASP method combined with a label-free quantification is an excellent proteomic approach for the analysis of fibrin clots prepared ex vivo from citrated plasma of patients with various diseases.

Keywords
proteomics, clot, venous thromboembolism, MED FASP

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Methionine-to-isothreonine conversion during sample preparation and its effects on proteomic data

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Background

Met-to-isoThr conversion was noted by us [Chernobrovkin et al., 2015] and nearly led to a false discovery of a mutation during proteogenomic study. It was hypostasized to be happening during the alkylation step of sample preparation as a result of iodoacetamide treatment. In the current work we are presenting an investigation of such modification in proteomic data and an experiment with serum albumin.

Methods

A few datasets taken from ProteomeXchange have been screened for Met-to-isoThr modifications using MaxQuant software and a detailed analysis of the result has been performed.
In the experiment, serum albumin has been treated with 30 and 50 mM iodoacetamide and also heating up to 95oC was applied. Then pseudo-MRM analysis was performed on Orbitrap mass spectrometer.

Results

In the data from [Moghaddas Gholami et al., 2013] 0.69-3.12% of Met were converted to isoThr. Interestingly, the neighborhood with Pro following Met seems to enhance the reaction of Met-to-isoThr conversion as the p-value of Fisher's Exact Test is 0.00001 with the correction for multiple comparisons, when the calculation was held with respect to all Met-Pro combinations in the peptides. On the other hand, Ser, following Met, seems to reduce the rate of the modification (p-value 0.04).
The result of the serum albumin experiment shows significant increase of the modification rate if the sample has been treated with 50mM comparing to 30 mM.

Conclusions

Met-to-isoThr conversion happens during sample preparation for proteomic analysis if high concentration of iodoacetamide is used, which makes the in-gel protocols prone for such event. This conversion is generally a rare event, though it might cause a false discovery in a proteogenomic workflow and, thus, should be taken into account. Proline following Met in peptides increases the probability of the modification and serine reduces this probability.

Keywords
methionine, isothreonine, proteogenomics, homoserine, sample preparation

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jPOST provides a global public data repository for a wide variety of proteomics experiments

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Background

Major advancements have recently been made in mass spectrometry-based proteomics, yielding an increasing number of datasets from various proteomics projects worldwide. In order to facilitate the sharing and reuse of promising datasets, it is important to construct appropriate, high-quality public data repositories.

Methods

Generally, researchers use different experimental methods for different experiments, although a single researcher usually uses a limited number of experimental methods. Thus, our repository also manages information related to the experimental procedures as a ‘preset’ and information specific to each experiment as a ‘project’. For the deposit of datasets, users are required to register their experimental details as presets. After the presets are registered, users create a project to deposit datasets and apply the configured presets to proteome data files.

Results

The jPOST repository has successfully implemented several unique features, including high-speed file uploading, flexible file management and easy-to-use interfaces. This repository has been launched as a public repository containing various proteomic datasets and is available for researchers worldwide. In addition, our repository has joined the ProteomeXchange consortium, which includes the most popular public repositories.

Conclusions

Our repository contributes to a global alliance to share and store all datasets from a wide variety of proteomics experiments. Thus, the repository is expected to become a major repository, particularly for data collected in the Asia/Oceania region. Our repository is available at https://repository. jpostdb.org/.

Keywords

jPOST, repository, database

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Affinity MS identifies pre-synaptic proteins SNAP-25 and synaptotagmin-1 as novel CSF biomarkers for Alzheimer's disease

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Background
Our objective is to identify cerebrospinal fluid (CSF) biomarkers of synaptic damage in neurodegenerative diseases. Synaptic loss occurs early in e.g. Alzheimer's disease, and measurements of altered synaptic function could be important to monitor disease progression and drug effects. The SNARE complex regulates synaptic neurotransmitter release and changed expression of SNARE proteins (SNAP-25, syntaxin-1 and VAMP) has been found to alter synaptic function. Another highly interesting possible target is the pre-synaptic vesicle protein synaptotagmin-1 which appears to be essential for the maintenance of an intact synaptic transmission and cognitive function. In this study we developed an affinity proteomics approach to quantify soluble forms of SNAP-25 and synaptotagmin-1 in clinically relevant volumes of CSF.
Methods
SNAP-25 and synaptotagmin-1 was co-immunopurified from 200 □LCSF samples on a KingFisher™Flex Purification System (Thermo Fisher Scientific Inc.). Stable isotope labeled peptide/protein standards were added before and during digestion and quantification was performed by high resolution parallel reaction monitoring mass spectrometry (HR-PRM-MS) on a Q Exactive (Thermo Fisher Scientific Inc.) coupled to an Ultimate 3000 standard liquid chromatography system (Thermo Fisher Scientific Inc.).
Results
We report a novel strategy to study several properties of synaptic pathology in parallel by combining affinity purification and proteomics to characterize and quantitate SNAP-25 and synaptotagmin-1 in CSF. A clinical cohort with CSF samples from patients diagnosed with early AD, prodromal AD, mild cognitive impairment, late AD, and non-neurodegenerative controls was analyzed with the SNAP-25/synaptotagmin-1 assay.
Conclusions
The strategy we present make it possible to compare levels of several soluble forms of synaptic proteins in individual patient CSF samples. This could be important for earlier diagnosis, assessment of disease progression, and to monitor drug effects in treatment trials.

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iST: a reproducible and universal sample preparation method for in-depth proteome discovery and interaction proteomics

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Background
Mass spectrometry (MS)-based proteomics typically employs multiple sample processing steps, representing a crucial part of routine MS analyses. Complex workflows, extensive sample fractionation and proteolytic digestion are highly time-consuming and restrict the technical reproducibility. The accuracy and robustness of the MS platform is also strongly affected by sample quality - reasoning for high quality proteomic samples. Here, we present the straightforward and robust in-StageTip (iST) method for streamlined sample processing of complete proteomes and immunoprecipitations (IPs).

Methods
The iST method is a 3-step procedure performed in a single, enclosed volume, which thereby circumvents the likelihood of contamination and sample loss. Due to the straightforward nature, the method can readily be performed in a 96-well format on liquid handling robotic systems. The method is highly compatible with StageTip-based pre-fractionation methods and thereby allows in-depth analysis of complex proteomic samples. Moreover, the iST method can be applied to immunoprecipitation samples or to clean-up samples with high detergent contamination.

Results
Applying the iST method to the well-studied cancer cell line HeLa allowed us to estimate protein copy-numbers of 9,667 proteins. The results demonstrated excellent reproducibility ($R^2 = 0.97$) in quadruplicates measurements, reflecting the overall strength of the method. In addition to cell lines, we applied this sample preparation workflow to process antibodies, yeast, tissue samples or body fluids such as blood, urine or CSF and obtained in-depth proteome coverage. Finally, we demonstrate the compatibility of the iST method with immunoprecipitation approaches utilizing agarose and magnetic beads, enabling streamlined and robust sample processing for IP samples. Our workflow retrieves known complex members and efficient enrichment of DNA-repair factors to a bait protein involved the base excision repair pathway.

Conclusions
The iST method opens up new perspectives for clinical applications and protein interactomics, while keeping exceptional sample quality at low-costs.

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The quantitative proteomic study of human saliva samples obtained from caries-free and caries-susceptible people

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Most people worldwide suffer from dental caries. Only a minor part of the population in the age of 30 is caries-resistant and reason of this resistance in unknown. Our study was aimed to proteomics of saliva and to find the differences in the abundances of the responsible proteins in these tissues between caries-resistant and caries-susceptible people. Only few studies compared the protein saliva composition of people with carious teeth and people with no caries (Vitorino, 2006; Preza, 2009). Proteomic analysis of human saliva revealed differences in protein expression according to gender and age (Fleissig, 2009).
Unstimulated whole saliva samples were collected from healthy females and males volunteers aged between 20 and 45 years (caries-resistant and caries-susceptible (more than five dental caries in their oral cavity)). The samples were centrifuged and were divided on two parts of samples: supernatant and pellet and then the proteins of oral fluids were separated by two-dimensional electrophoresis and by Label-Free Quantitative analysis. The resulting protein maps were quantitatively evaluated. Spots exhibiting statistically significant changes were excised and analyzed by nano-liquid chromatography coupled to Q-TOF mass spectrometer (MaXis, Bruker).
We detected the significant differences in the protein composition between the samples of saliva separated from caries-susceptible and caries-resistant females and males.
Our result demonstrates that the observed differences in the protein levels might have influence on the anti-caries resistance. This result should be further verified on a larger group of respondents.
The study was supported by the Charles University in Prague, project GA UK No. 322216.

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Global profiling of dehydration-induced mitochondrial dynamics and defense response in rice

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Background
Elucidation of molecular basis of dehydration induced cellular combat not only aids understanding of plant biology, but has direct implications towards fortification of sustainable agriculture. Previously, we successfully established stress-responsive proteome map of nucleus and cell wall in rice. Next we focused on mitochondria, which serve as dynamic microenvironment for integration of cellular metabolism and signalling towards understanding energy metabolism under water-deficit conditions.
Methods
Four-week-old rice seedlings were subjected to progressive dehydration and stress severity was assessed. Further, integrity and purity of isolated fraction was evaluated. Identification of proteins was carried out at 1% FDR. Metabolic profiling was carried out in Thermal Desorption System (TD 20) comprising an AOC-20i auto-sampler connected to GC-MS. Graphical and statistical analyses were performed on the data with a one-way analysis of variance (ANOVA) and Duncan's Multiple Range test (DMRT) using GraphPad Prism V 7.00.
Results
In-depth comparative proteomics analysis using iTRAQ led to identification of 101 significantly altered proteins, presumably involved in a variety of cellular functions. Further, metabolite profiling and network analysis revealed high abundance of branched chain amino acids and sugars under water-deficit condition seemingly to reduce osmotic potential. The proteo-metabolomic profile demonstrated that alteration of tricarboxylic acid cycle intermediates is crucial for fuelling ATP production. A critical screening of mitochondrial proteome revealed an unknown protein, putatively associated with calmodulin-related calcium sensor, having Domain of Unknown Function. Study of differential transcript regulations revealed strong dehydration-induced mRNA abundance of this protein. Dehydration-induced upregulation was consistent with its altered expression as revealed by proteome analysis, suggesting its function in dehydration adaptation.
Conclusions
The present study ascertained a comprehensive view of effects of dehydration on mitochondria of rice, world’s most valuable food grain. Dehydration responses of the seedlings were integrated with molecular networks using proteo-metabolomic data.

Keywords
Mitochondria, sustainable agriculture, dehydration, iTRAQ, metabolite profiling

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Dynamic composition of the Cryptococcus neoformans proteome and secretome during dormancy phenomenon

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Latency is an important phenomenon in the pathophysiology of Cryptococcosis. Evidence for dormant cells of Cryptococcus neoformans was obtained in mice and an in vitro model was implemented in our laboratory. We hypothesized that diffusible factors could be secreted into the medium, allowing induction/maintenance of dormancy and viability. We aimed to identify the proteins secreted in the supernatant during induction of dormancy in parallel to the analysis of the whole yeast cell proteome.

C. neoformans proteins were isolated from the supernatants and the yeasts pellets at different time points in anaerobiosis and aerobiosis. Proteins were digested and resulting peptides were analyzed on a Q Exactive Plus coupled to a nanoLC system (3 hours gradient; C18 column, 50 cm). Data was processed with MaxQuant, Perseus and in house R packages.

More than 56 % of the predicted proteins were identified from both secreted and whole proteome analysis.While the number of identified proteins in the proteome was stable irrespective of the culture condition, the anaerobiosis condition was associated with a decrease of secreted proteins over time. Exclusive secreted proteins and more abundant proteins from anaerobiosis were selected for downstream analysis. The biological function of proteins was assessed with a GO term enrichment analysis comparing each secreted protein to the complementary whole cell extract. Furthermore, the role of selected proteins was investigated using available KO mutants from Fungal genetics Stocks Center. Eight KO mutants showed no viability in anaerobiosis, suggesting that the corresponding proteins are necessary for the maintenance and viability of yeast.

This work allows us to gain insights into the process of inducing and/or stabilizing the state of dormancy in C. neoformans by revealing new key factors. Further validation using KO mutant libraries demonstrated that some of the identified proteins were necessary for the maintenance of yeast viability.

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Analyzing the mechanisms of acquired resistance to MEK inhibitors in colorectal cancer using multi-proteomics approaches

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Background
BRAF and KRAS mutations occur in approximately 10% and up to 50% of colorectal cancer (CRC) patients, respectively. Those alterations lead to constitutive activation of signaling downstream of epidermal growth factor receptor (EGFR), including the Ras/Raf/MAPK/MEK/ERK and/or PTEN/PI3K/Akt pathways. Thus treatments based on anti-EGFR antibodies are ineffective in patients with advanced CRC. MEK inhibitors became novel drugs in those therapies and have shown great effectiveness. Although MEK inhibitors have recently shown promising results in early CRC trials, resistance invariably occurs. Therefore the investigation of the underlying mechanisms is crucial for designing new treatment regimens.
Methods
BRAF and KRAS mutated CRC cell lines were treated with increasing Trametinib concentration over the course of 4-8 months and lysed when showing substantial resistance to the initial growth inhibitory drug effect. We analyzed kinases expression level after enrichment on Kinobeads, full proteomes after fractionation on Trinity P1 and phospho-proteomes enriched on IMAC column and fractionated by high pH reversed phase. Label free (Kinobeads) and TMT-6 labeled tryptic peptides (full- and phospho-proteomes) were analyzed on a QExactive HF mass spectrometer. Identification and quantification of proteins was performed using MaxQuant with subsequent analysis in Perseus.
Results
Cell lines cultured in the presence of Trametinib showed a 20-2000 fold increase in resistance relative to the corresponding parental cell line (EC50resistant/EC50parental). We identified a median of 5867 protein groups, 173 kinases and 4380 of phospho-sites per cell line. Preliminary results are pointing to BRAF overexpression and EPHA2 up-regulation as important mechanisms in Trametinib resistant COLO205 cell line. Many additional mechanisms of acquired resistance like MAP2K/p38 bypass pathway activation were detected in SW480 and SW620 cell lines.
Conclusions
This data implies that kinase inhibitor combination treatments could be an interesting option for future treatment of MEK inhibitor resistant patients.
Keywords
Acquired resistance, MEK inhibitor, Trametinib, CRC

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iTRAQ labeling and label-free quantitative proteome analysis of synaptosome from schizophrenic patients

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Background: Schizophrenia (SCZ) is a neuropsychiatric disorder characterized by impairment of mental functions through the manifestation of symptoms like delusions, hallucinations, anhedonia and social withdrawal with deficiency in cognitive processes. The pathophysiology of SCZ is not completely understood and the characterization of disease-associated brain proteins from the synaptosome fraction of patients with SCZ, using a quantitative proteomics approach, will be helpful to elucidate the SCZ pathogenesis.
Methods: Synaptosome fraction of post-mortem brain tissues was prepared from the orbitofrontal cortex region of SCZ patients (n=6) and controls (n=8) following Gray & Whittaker's protocol. Extracted proteins were digested and iTRAQ labeled using three reporter groups for patients and one for a pool of controls. Labeled and label-free peptide mixtures were analyzed in an EASY-1000 nanoLC coupled to Orbitraps Velos and Q Exactive Plus, respectively. Mass spectra were analyzed in Proteome Discoverer 2.1 against the NeXtProt and Uniprot (FDR <1%); for statistical analysis was used the Inferno RDN software.
Results: MS analyses identified 737 and 1906 protein groups by iTRAQ labeling and label free analysis, respectively. Considering at least 3 unique peptides, 129 and 358 proteins were consistently quantified. The altered proteins are predominantly involved in processes such as: 1) Neuronal development related to the regulation of neurite growth; with the increase of proteins like LSAMP and Alfa-internexin 2) Dysregulation of proteins involved in the release of synaptic vesicles, such as calmodulin and alpha-synuclein 3) Myelin sheath formation like myelin basic protein, proteolipid protein and myelin oligodendrocytic glycoprotein.
Conclusion: Implementation of iTRAQ and label free techniques allowed a deep and complementary quantitative proteome analysis, revealing the dysregulation of proteins associated with neurite growth, synaptic vesicles and myelin sheath formation contributing to a better understanding of SCZ pathophysiology.
Keywords: Schizophrenia, synaptosome, quantitative proteome, pathophysiology

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Benchmarking data-independent acquisition workflows using hybrid proteome samples and LFQbench

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Background: In mass spectrometry-based proteomics, multiple factors including the reproducibility of sample preparation, instrument type, acquisition method and analysis software influence the reliability of experimental results. To measure the accuracy and precision of mass spectrometry-based quantification workflows, the analysis of spiked protein or peptide standards into complete ''background'' proteome is a widely accepted method. However, as spiked standards can only provide a limited number of data points for evaluation, this technique provides a limited statistical power. To overcome this limitation, we recently developed a novel workflow for benchmarking proteomics analysis systems based on the mass spectrometric analysis of a complex hybrid proteome sample set and the subsequent evaluation of quantification results using the open-source software package LFQbench. In a previous study, we applied this benchmarking strategy to evaluate multiple SWATH-MS data analysis workflows, enabling developers to improve their software tools and providing the community with high-complexity hybrid proteome reference datasets.

Methods: The hybrid proteome sample set is composed of digested complete proteomes of three different species (Human, Yeast, E.coli) in predefined ratios simulating non-regulated (background), down- and upregulated proteins. Utilizing the a priori knowledge of ratios for each protein of a species, LFQbench calculates a set of metrics for the accuracy and precision of label-free quantification. We analyzed hybrid proteome samples using multiple data-independent acquisition techniques (SONAR, HybridSONAR and UDMSE) and applied LFQbench to benchmark the resulting datasets.

Results: To demonstrate the flexibility and power of the approach, we compare current and previous benchmark results to discuss advantages and benefits of the different data-independent acquisition techniques.

Conclusions: The datasets produced in this study align perfectly with the community efforts to establish a reach set of reference proteomics datasets allowing for increasing the consistency and comparability in studies evaluating and benchmarking different instruments, acquisition techniques or analysis software tools.

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Identification of cross-linked peptides in proteins subjected to photo-oxidation

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Background:
Protein cross-links are ubiquitous in biological systems and they can be generated in regulated biological processes, but also through reactions with exogenous oxidants. Identification of such cross-links using mass spectrometry is still very challenging due to their undefined chemistry. The goal of this study was to develop a workflow aimed at identifying and characterizing cross-links in protein samples subjected to free radical oxidation using optimized ^{18}O isotopic labeling, different types of mass spectrometry acquisition workflows, and a designated database software tool.

Methods:
Selected proteins including lysozyme C, glucose 6-phosphate dehydrogenase and RNase A were cross-linked via a photo-oxidation process using Rose Bengal or incubation of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Proteins were digested with trypsin in the presence of either ^{16}O water or ^{18}O water, mixed and analyzed using LC-MS/MS (Orbitrap Fusion, Thermo Fischer) using three acquisition methods. Data analysis was carried out both by manual spectral interpretation and using different software including MaxQuant, GPMAW and MassAI. A Lactococcus lactis protein extract exposed to AAPH oxidation was also used in order to evaluate this approach for cross-linking identification in more complex samples.

Results:
By applying this workflow, different types of cross-linked peptides were identified, including tyrosine-tyrosine, tyrosine-tryptophan and tyrosine-lysine cross-links. The presence of these cross-links is indicated by the 8 Da shift in the MS spectra due to the ^{18}O labeling and also by the loss of 2 Da in the cross-linked peptide mass due to the nature of these particular cross-links.
Different fragmentation techniques were also evaluated, including HCD, ETD and EThcD. It was shown that EThcD fragmentation provided higher sequence coverage of cross-linked peptides, while HCD resulted in higher number of identified cross-links.

Conclusions:
Several novel protein cross-links with undefined chemistry have been successfully identified using this mass spectrometry-based workflow.

Keywords:
LC-MS/MS, ^{18}O labeling, free radical oxidation, cross-linking

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Comparative proteomic study of affected and non-affected tissues of clubfoot

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Background: Congenital talipes equinovarus (CETV), also referred as clubfeet, occurs in 1 to 2 in 1000 live births, and is one of the most common birth defects involving the musculoskeletal system [1]. The pathogenesis of this disease is unknown despite numerous hypothesis.
Methods: We compared the capsule specimens of talo-navicular joint on medial (affected) side of the foot (M-side of foot) with normal tissue at the plantar surface of the calcaneocuboid joint (non-affected lateral side; L-side) of thirteen patients with CTEV (patients undergoing surgery for clubfeet). The extracted peptides were analyzed, and compared by label-free MS (nano-liquid chromatography coupled to a maXis Q-TOF mass spectrometer).
Results: In total we detected significant increases in 11 protein concentration in affected tissue (M-side) of clubfoot disease (apolipoprotein A I; asporin; collagen types III, V, and VI; decorin; hemoglobin subunit alpha and beta; immunoglobulin heavy chain; prolargin; tenascin; TGFβip) in comparison with L-side. Most of these proteins are incorporated into tissue remodeling and/or fibrosis. In the other side we detected 4 significantly upregulated proteins in L-side (cartilage intermediate layer protein; collagen types XII and XIV; fibromodulin). Also we indirectly confirmed upregulation of TGFβ□in M-side in compartison with L-side - this protein has been observed in higher concentration in connection with this disease before [2].
Conclusions: This study opens new view to clubfeet disease by complex comparative proteomic analysis and brings new alternative targets, which are closely connected to these fibrosis processes.

Keywords: Pes equinovarus, clubfoot, proteomics, fibrosis, label-free quantification, orthopedics, pediatrics

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HLA ligandomics drives the identification of targets for immunotherapeutics based on soluble T-cell receptors

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Background
HLA complexes present peptides derived from cancer-associated proteins to the immune system that can be used as targets for cancer immunotherapy. Immunocore identifies HLA peptides, generates T-cell clones with reactivity towards these peptides and engineers T-cell receptors (TCRs) from these clones into potent soluble immunotherapeutics.

Methods
Identification and selection of target peptides is paramount to the success of effective TCR-based drugs. Selection criteria are based on differential expression between cancerous and normal tissues, coupled with the identification and relative quantification of HLA peptides in cancer cell lines and tissues using advanced mass spectrometric technologies.
Fully validated peptide targets are used to clonally expand HLA-peptide specific T-cells. TCRs isolated from these cells are engineered into soluble molecules (mTCRs) whose affinity toward target peptide:HLA is increased using phage display technology.

Results
Our HLA peptidomic workflow couples novel biochemical techniques with high resolution mass spectrometry and is designed to maximise the depth of the HLA ligandome captured. Our workflow includes the use of advanced, decision-guided acquisition methods and the integration of data from multiple instruments and multiple search algorithms.
Our immune-activating therapeutics (ImmTAC™) are generated by coupling high-affinity mTCRs with an anti-CD3 scFv domain. Anti-CD3 mediated T-cell redirection is a clinically-validated, highly potent therapeutic mechanism of action. ImmTAC molecules activate polyclonal T-cell responses targeted toward cells presenting the appropriate cancer-associated peptides. Our current lead candidate, IMCgp100, is currently undergoing Phase I/II clinical testing as a monotherapy for the treatment of patients with advanced uveal melanoma and in combination with checkpoint inhibitors for patients with metastatic cutaneous melanoma.

Conclusion
Mass spectrometry-based peptidomics enables the identification of HLA-presented peptides to support the successful design of TCR-based immunotherapeutics for cancer treatment.

Keywords
Human-Leukocyte Antigen; T-cell receptor, Mass spectrometry, Ligandome, Immunotherapy

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Novel Osteoporosis Risk Protein Biomarkers Suggessted by a Proteome Study in Chinese Elderly Men

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Osteoporosis is characterized by low bone mineral density (BMD) and progressive microarchitectural deterioration of bone tissue, leading to increased risk of osteoporotic fracture (OF).Peripheral blood monocytes(PBM) can differentiate into osteoclasts .With case-control design, the present study aims to identify OP risk proteins in Chinese elderly men.
We recruited hip OF patients (n=18, age mean: 77 yrs) and subjects without fracture history (abbreviated as NF, n=18, age mean: 73 yrs). The NF subjects included extremely low BMD and high BMD subjects (hip Z-score: -1.06 vs. +1.36). PBM were isolated from peripheral blood and total proteins were extracted. Proteome-wide protein expressions were profiled by employing label-free quantitative proteomics methodology.Proteomic data were processed by the softwares Maxquant and Perseus to identify differentially expressed proteins (DEP, $p \leq 0.05$, FoldChange≥2.0).Bioinformatic analysis were conducted to annotate functions of DEPs.With regard to DEPs of interest, plasma proteins levels were quantitated by ELISA and tested for association with OF.
Sixty one DEPs were identified between subjects with low and high BMD and they were significantly enriched in biological processes, including "leukocyte migration" (p=6.73E-04), chemotaxis (p=0.01).AKT1 protein exclusively expressed in high BMD subjects was significantly enriched in the Osteoclast Differentiation pathway (KEGG ID 04380).Specifically, the ABI1 protein expression was down-regulated in low BMD vs. high BMD subjects (FC=0.82,p=0.043). The same regulation tendency was observed in OF subjects vs. NF subjects (FC=0.73,p=0.008).In addition,an extracellular protein SRC was quantitated in plasma by ELISA between OF and NF subjects. SRC protein, which was down-regulated in PBM in OF subjects in contrast to NF subjects (FC=0.45,p=0.0009), was validated to be down-regulated in OF subjects in plasma as well (OF vs. NF : 40 vs. 44,FC=0.55,$p<0.001$).
In conclusion, the present study identified novel osteoporosis-related proteins, e.g., AKT1,ABI1 and SRC . Whether the DEPs are predictive of osteoporosis has yet to be evaluated inlongitudinal study cohort.

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GLP-1 analogues modulate MAPKs activity but not the heat shock response in human HepG2 cells

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Purpose: Glucagon like peptide-1 (GLP-1) analogues reduce ER stress and inflammation in key metabolic organs including liver. However, their effects on heat shock response (HSR) and MAP-Kinases (MAPKs) have not yet been elucidated. We investigated whether GLP-1 analogue; Exendin-4, triggers the expression pattern of HSR and MAPKs activity under metabolic stressful conditions.
Experimental design: Western blots, quantitative real-time PCR and label-free proteomics were used to assess the effects of Exendin-4 with and without palmitic acid (PA, 400uM) or Glucose (30mM) on HepG2 liver cell line.
Results: Heat shock proteins (HSP60, HSP72, HSP90 and GRP78) and other chaperones were not significantly affected by Exendin-4 regardless the used conditions. By contrast, the phosphorylation levels of MAPKs (JNK, ERK1/2 and p38) were clearly increased in the presence of Exendin-4 alone. In the presence of PA or glucose, Exendin-4 displayed limited effect for short incubation periods but significantly attenuated MAPKs phosphorylation after 24h incubation. Interestingly, canonical signalling pathways such as EIF2, ILK, PKA and Rho were clearly modulated by Exendin-4.
Conclusion and clinical relevance: Identifying new pathways modulated by GLP-1 analogues provides insight into its beneficial effects beyond glycaemic control, such as MAPKs activity, energy homeostasis in glucose up-taking organs and body weight decrease.

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QCloud: An automated community cloud-based system for quality control in proteomics experiments

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Background
Increasing number of biomedical and translational applications presents the proteomics community with new analytical challenges and rises the need for automated quality control systems. Despite previous efforts to set standard file formats, data processing workflows and key evaluation parameters for quality control (QC), automated QC systems are not yet widespread among proteomics laboratories. The lack of robust QC pipelines that systematically monitor instrument performance limits high-quality results, inter-laboratory comparisons and the assessment of variability of instrumental platfoms. Here we present QCloud, a cloud-based system to support proteomics laboratories in daily QC monitoring using a user-friendly interface, easy setup, automated data processing and archiving, and unbiased instrument evaluation.

Methods
A cloud-based QC system has been developed based on Java, OpenMS, the qcML data format, and a LAMP webserver. A thin client installed in the instrument computer locates and uploads QC sample acquisition files and instrument parameters through a remote FTP server. QC samples are then processed for spectra assignment and peptide area extraction, QC parameters are stored in the persistence layer and data is presented in a web front-end based on jQuery and Google Charts. Statistical assessment of QC data establishes acceptance thresholds and enables automated and unbiased instrument performance evaluation.

Results
A fully functional version of the QCloud has been successfully developed and subsequently deployed within the Core for Life proteomics partner laboratories (https://coreforlife.eu/) for over a year, and is now open to the entire proteomics community (qcloud.crg.es). Currently the system supports all most common proteomics workflows, including shotgun discovery analysis, PRM, SRM as well as MS1-based quantitation. Moreover, the system accepts data formats from different mass spectrometer vendors and enables user selected vocabulary to report incidences and annotate the acquired data.

Conclusions
We present an automated cloud-based system for systematic and unbiased instrument evaluation for the proteomics community.

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Membrane Proteome Profiling of Peripheral Immune Cells in Non-small-cell Lung Cancer Patients with PEM/CIS Chemotherapy

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Background
Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer worldwide. Currently, pemetrexed (PEM), combined with cisplatin (CIS), has been used as the first-line treatment for advanced NSCLC patients carrying wild-type EGFR. The impact of PEM/CIS has been reported in tumors as well as in several specific populations of immune cells. In this study, we aim to unravel the overall immune status in membrane proteomic scope of view on NSCLC patient upon PEM/CIS treatments.
Methods
Paired PBMCs from 4 patients were collected before and after first regimen of PEM/CIS treatment for individualized analysis of membrane proteome profiles. The membrane protein fractions were purified from PBMCs, subjected to gel-assisted digestion, labeled with iTRAQ reagents, fractionated by high-pH reversed phase StageTip, and analyzed in duplicate by LC-MS/MS. Protein identification was obtained by using Mascot with 1% false discovery rate. Protein quantitation was applied by using Multi-Q.
Results
A total of 2,424 proteins were identified and the Pearson correlation analysis revealed diverse and patient-specific patterns of the PBMC membrane proteome profiles. To determine the patient's immune status after PEM/CIS treatment, we examined the expression levels of marker proteins/pathways and discovered a more suppressive T cells phenotype with down-regulation of CTLA4 degradation in patient with better progression free survival (PFS) of 14.5 months. Besides, neutrophil elastase and myeloperoxidase were upregulated, suggesting elated neutrophil activity. On the other hand, patients with 2-month PFS possessed overall higher expressions of T cell subsets and MHC II pathways which indicated an activated immune status.
Conclusion
Our study demonstrated that PEM/CIS therapy altered patient's immune system in neutrophil, T cells, and antigen presenting pathways and provided overall drug-immune interactions without additional isolation of specific immune cell populations.
Keywords
Quantitative membrane proteomics, PEM/CIS, peripheral immune cells, NSCLC

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Development of an apoE-isoform specific PRM assay aiming early prediction of Alzheimer's disease.

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Background:
Alzheimer's disease (AD) is the most common form of dementia and accounts for ~50 Mio cases world-wide. Clinically validated biomarkers for early prediction of AD are lacking. Apolipoprotein E-variant APOE4 is a strong genetic risk factor, and potential clinical relevance of serum-levels of total apoE and individual isoforms are debated controversially in literature. Here, we tested the hypothesis if serum concentrations of the three most common isoforms of apoE are associated with future risk of AD.

Methods:
A matched case-control set of 300+ samples from the Rotterdam Scan Study was analyzed (~100 cases that developed AD; ~200 non-demented matched controls). High-throughput sample preparation was conducted in well-plates and included spiking of SIL standards, denaturation, digest and chemical modification of cysteine and methionine (quantitative oxidation; necessary to quantify isoform-specifying peptides that contained methionine). LC-MS measurements were run on an Orbitrap Fusion in parallel reaction monitoring mode. Assay development and evaluation was conducted, if applicable for proteins, following EMA and FDA guidelines.

Results:
Assay developed and evaluated enables measurement of serum-levels of the three most-common apoE-variants; cysteine and methionine containing peptides were successfully included. Serum-levels of apoE and correlation between phenotypes and serum levels were in agreement with results of other studies published. No significant association between risk of AD and serum-levels of the different apoE isoforms were found after adjustment for age, gender, and phenotype. Ratio of apoE2 and apoE4 relative to total apoE was on mean 59% (sd=5%) and 46% (sd=7%), respectively, and phenotype dependent differences in total apoE originated from expression changes of all present isoforms.

Conclusions:
Serum-levels of total apoE, apoE2, apoE3, and apoE4 as well as the stoichiometric ratios between these isoforms could not confidentially predict risk of AD in this matched case-control study.

Keywords:
Alzheimer's disease (AD), phenotyping, parallel reaction monitoring (PRM), EMA/FDA-guidelines, population study

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Identifying oxidation-specific post-translational modifications during diet-induced liver inflammation

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Background
Oxidative processes are a hallmark of many chronic inflammatory diseases. Increased oxidative stress leads to the generation of highly reactive species that can irreversibly modify proteins and other biomolecules. There is accumulating evidence that oxidative modification of proteins renders them immunogenic and can therefore lead to the perpetuation of an inflammatory process. However, there is little information on the exact epitopes that trigger immunogenicity. Although many oxidation-specific epitopes are targeted by specific antibodies, the exact identity of these epitopes is largely uncharacterized on the molecular level.
Methods
We are applying an unbiased screening approach to identify previously unrecognized oxidation-derived post-translational modifications (PTMs) that may act as mediators of liver inflammation. LDL-receptor KO mice susceptible to diet-induced dyslipidemia are fed a high fat diet, which triggers hepatic inflammation. Livers are subjected to proteomic analysis on an Orbitrap Fusion Lumos Tribrid mass spectrometer, and PTMs are identified using a high-tolerance database search (Chick et al, Nat. Biotechnol. 2015). The main advantage of this approach is that there is no need to have any a priori knowledge of expected PTMs, and therefore it is able to identify novel modifications.
Conclusion
By applying the tolerant database search we were able to identify a set of post-translational modifications that were more abundant in inflamed liver tissue from high fat diet-fed mice than in liver tissue from control mice. In addition, some of these candidate modifications exhibit specific binding towards selected natural IgM antibodies that had been previously cloned for the ability to bind different oxidation-specific epitopes. It is noteworthy that most candidate modifications appear to be derived from reactive aldehydes arising from lipid peroxidation. In conclusion, we provide direct evidence of oxidation-specific epitopes in hepatic inflammation that have the potential to modulate the immunological response.
Keywords
Post-translational modification, hepatic inflammation, oxidative stress, lipid peroxidation

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Verification of saliva proteins candidates for OSCC markers and their correlation with prognosis

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Background. The most common oral cancer in the world is squamous cell carcinoma (OSCC), accounting for more than 90% of all cases of cancer in the oral cavity. Thus, the research on molecular markers associated with the development and progress of human diseases has been the subject of intense research. The findings that saliva has molecular profiles indicating systemic diseases urge the study of non-invasive diagnosis using saliva as source of potential diagnosis, prognosis and predictive based on proteomics.
Methods. 114 proteotypic peptides were selected previously based on DDA data from our own studies or retrieved from the SRMAtlas. Heavy labeled synthetic peptides were synthetized and MRM method was developed using Skyline v3.6. Saliva samples were collected from patients with lymph node metastasis (n=20) and without lymph node metastasis (n=9). Saliva proteins were digested with trypsin. Samples were randomized in R environment and analyzed in a triple-quadrupole mass spectrometer (Xevo TQ-XS, Waters). A Peptide Retention Time Calibration Mixture (iRT, Pierce, Thermo) was spiked into the samples. Data comparison between groups was performed in Skyline.
Results. In the MRM method, we monitored 642 transitions of 44 proteins and ten proteins were found increased in patients with lymph node metastasis in comparison with patients without lymph node metastasis.
Conclusions. This study indicates a panel of potential OSCC marker proteins, which can contribute to the prognostic evaluation, the patient's risk profile and the possibility of recurrence, and guide therapeutic intervention strategies.

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Proteomic analysis of serum from patient with alcoholic pancreatitis to compare disease with treated status

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Background

Acute pancreatitis (AP) is a sudden inflammation of pancreas. There are various causes of AP, but most common causes are alcohol, gallstones and hyper-triglyceride. Approximately 80 % of the patients showed the mild clinical course of AP, but about 10% of AP showed severe clinical aspect with multiple organ failure such as acute kidney and lung injury, leading to increased mortality. Therefore, it is important to accurately predict severe AP in early stage and to select patients who are expected progress to severe clinical aspect. We performed a proteomic analysis to discover specific diagnostic markers in patients with alcohol-induced AP.

Methods

Serum samples (n=16) were collected during hospital days, and select serum for proteomic analysis collected at hospital admission and before discharged from hospital. A multiple affinity removal system column was used for removal of high abundant proteins. Only low abundant proteins were prepared for LC-MS/MS analysis.

Results

Serum proteins of each patient were analyzed by LC-MS/MS, and an average of 265±46 proteins were identified in disease status and 310±39 proteins in treated status. Comparative proteomic analysis was performed by label-free quantification. In each patient, more than two fold changed proteins were identified. The total 16 proteins showed the same pattern in more than 50% of patients by MPP analysis. Up-regulated proteins of disease status were three: complement factor H-related protein 1, serum amyloid P-component, and c-reactive protein. Thirteen proteins including complement C1r subcomponent decreased in disease status.

Conclusions

Up-regulated proteins in disease status are highly associated with inflammation response. Down-regulated proteins are associated with oxidative stress and ECM organization as well as immune response. In a further study, we will verify the potential diagnostic marker for alcoholic pancreatitis by quantifying the identified proteins in the serum of individual patients using MRM.

Keywords

Acute pancreatitis, Alcohol, LC-MS/MS, Label-free quantification

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Novel CSF Tau Fragments as Candidate Biomarkers for Alzheimer's and other Neurodegenerative Diseases

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Background
Tau pathology is a hallmark of several neurodegenerative diseases, including Alzheimer’s disease (AD). Standard cerebrospinal fluid (CSF) total-tau immunoassays bind to the mid-region measuring increased levels in some, but not all neurodegenerative diseases. Novel tau cleavage sites have been identified in brain tissue but CSF data is limited and no cleavage-specific antibodies are available. We hypothesise that secreted fragments of tau may reflect disease specific cleavages of tau.

Methods
Tau was immunoprecipitated (IP) from human CSF using mAb’s directed towards different tau regions. “Bottom-up” and “top-down” analysis of digested and non-processed samples, respectively, were performed by nanoflow liquid chromatography–high resolution mass spectrometry (MS). End-specific antibodies were generated using designed tau peptides for immunization. IP-MS analyses of tau confirmed mAb specificity. ELISA or Simoa assays were developed and assay accuracy, precision, and limit of detection were evaluated.

Results
More than 80 endogenous tau fragments were detected by IP-MS. Three novel main cleavage sites on CSF tau were identified occuring at the N-terminal, mid-, and C-terminal region. Several monoclonal anti-tau antibodies, end-specific for the novel fragments, were obtained. mAb specificity was confirmed and ELISA or Simoa assays were able to quantify the tau fragments in CSF down to low pg/mL. Preliminary results suggest that the tau fragments may represent different pools of tau and that some may have diagnostic information.

Conclusions
Mapping of the CSF tau fragment pattern is vital to understand tau processing and its relation to neurodegenerative diseases. IP-MS analysis allows for identification of novel fragments of tau in CSF. Pilot studies confirm the feasibility of analysing these fragments in patient materials. Initial clinical validation demonstrates a potential diagnostic value for some fragments. The results presented will add to our current understanding on tau processing and tau pathologies.

Keywords
Alzheimer’s disease; cerebrospinal fluid; tau protein; mass spectrometry; immunoassay

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2D-precursor selection for trapped ion mobility with parallel accumulation - serial fragmentation (TIMS-PASEF)

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Background: With the previously introduced "Parallel Accumulation - Serial Fragmentation" method (PASEF, Meier et al., JPR 2015, PMID:26538118) for ion mobility (IMS) quadrupole time of flight (QTOF) instruments, five to ten times faster data dependent acquisition of fragment ion spectra became possible. This approach requires a fast two-dimensional (2D) precursor selection algorithm using mass as well as ion mobility information.

Methods: In TIMS-PASEF mode peptide ions elute from the IMS device as condensed packages. For most efficient MSMS acquisition the quadrupole isolation window needs to switch its isolation position exactly synchronized to these elution times in the fastest possible order. The corresponding algorithm was developed and evaluated using tryptic digests of HeLa cell lysates, separated by 90min nanoLC gradients. Data were analyzed using DataAnalysis (Bruker), Mascot (www.matrixscience.com), and MaxQuant (Cox group, MPI of Biochemistry).

Results: The precursor selection algorithm detects m/z and mobility positions of all precursors in the MS1-IMS scan. Then they are scheduled for measurement across multiple (ten to twenty) consecutive IMS experiments aiming for most efficient utilization of measurement time. Low intensity precursors are measured multiple times to achieve sufficient spectra quality. Elution length of an individual precursor ion is dependent on IMS resolution which is a function of mobility scan time. For tryptic peptides, mobility scan times of 25, 50, 100 and 200 ms resulted in average mobility resolutions between 20 and up to 80 and elution lengths between 1.8 and 10.6 ms. With a 100 ms IMS separation up to 900000 individual PASEF-MSMS spectra can be acquired during a 90min Hela nanoLC-run, resulting in about 200000 unique MSMS spectra after combining repetitive measurements.

Conclusions: We developed a 2D-precursor selection algorithm for TIMS-PASEF that determines and schedules precursors in complex samples within 100-200 ms, well suited for an LC-timescale.

Keywords: PASEF, TIMS, QTOF, data-dependent acquisition

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Proteomic and phosphoproteomic analysis of cells lacking the catalytic activity of protein kinase CK2

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Background
Protein kinase CK2 is a tetrameric holoenzyme composed of two catalytic (α and/or α’) and two non-catalyticβ subunits. CK2 is an extremely ubiquitous, constitutively active and acidophilic Ser/Thr kinase. More than 300 physiological substrates of CK2 are known and, based on its consensus sequence (S/T-X-X-E/D), CK2 has been considered responsible for the generation of about 20% of the entire phosphoproteome. Abnormally high CK2 level/activity is often associated with malignancy and a varietyof cancer cells are known to rely on this kinase to escape apoptosis.
Methods
To dissect CK2-dependent cellular processes we exploited the CRISPR/Cas9 technology to generate clones of C2C12 myoblasts lacking both catalytic subunits of CK2. A SILAC-based proteomics and phosphoproteomics analysis was performed to compare protein expression levels between wild type cells (WT) and two different clones in which CK2 activity has been abrogated.
Results
The SILAC analysis led to reliable quantification of more than 1400 proteins, 20% with altered abundance in the clones with respect to the WT. The phosphoproteomic analysis led to the identification of 1930 unique phosphopeptides, corresponding to 471 unique quantified phosphosites.
Conclusions
SILAC analysis of CK2α/α’(−/−) versus WT cells shows that knocking out both CK2 catalytic subunits causes a profound reorganization of the cellular proteome. Up- and down-regulated proteins fall into distinct sub-cellular compartments and play different biological roles, consistent with a global rewiring underwent by the cell to cope with the absence of CK2.
Our data reveal that only a minority of the phosphosites with a consensus sequence recognized by CK2 are affected by its absence, suggesting that other kinases could act as “substitute” for CK2 in the CK2α/α’(−/−) adaptation. Moreover, we show that phosphoproteomics data might be wrongly interpreted if a normalization based on the total amount of protein is not performed.
Keywords
CK2, Phosphoproteomics, CRISPR/Cas9, Normalization, kinase.

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Reducing false positive identifications for proteome datasets accumulated in jPOST repository

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Background
As is now widely known, accumulating search results from multiple projects and institutions leads to linear increase of false positive hits. To avoid this problem, our jPOST (Japan ProteOme STandard Repository/Database) repository as a member of ProteomeXchange Consortium has accepted MS raw data files in addition to the search results to minimize the false discovery rates for the accumulated datasets. Multiple search engines as well as multiple peak picking algorithms are employed for the raw files to reduce the false negative hits in the first step. In this presentation, we will report the second step to minimize the false positive identifications.

Methods
MS raw data acquired in the global proteome analysis of Fibroblast_cell_aHDF1388-P9 were obtained from the jPOST repository with PXD004620. The peak list was created by ProteinPilot 4.5 and the protein identification was performed by X!Tandem 2015.04.01.1 against UniProt 2016_3. The decoy database was generated by randomizing sequences from UniProt.

Results
We applied the concept of peptide sequence tags (PSTs) to the peptides identified both from the forward and the decoy databases to evaluate the quality of MS/MS spectra. As a result, we found that it was very rare for decoy hits to have sufficient sequence tags even when the original peptide score is high enough for identification. Based on these findings, we set the PST-based properties for ranking the peptides. By PST-based sorting the peptides originally identified by X!Tandem, the highest rank of the decoy hit dropped from the rank 6,202 to 7,020 in 10,882 assignments, and the number of identified peptides at FDR 0.1% was increased from 7,608 to 8,381.

Conclusions
We successfully reduced false positives in protein/peptide identification by utilizing PST-related properties. This approach is applicable for re-analysis of accumulated datasets in public repository such as jPOST.

Keywords
jPOST, database, reanalysis, FDR, PST

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Phosphoproteomics analysis of plasma from Vietnamese type 2 diabetes patients

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Protein phosphorylation plays a central role in cellular processes such as cell cycle, cell growth, apoptosis, and signal transduction. An abnormal regulation of protein phosphorylation may result in a number of dysfunctional cellular processes that lead to various diseases. There are evidences indicating the relationship between phosphorylation/dephosphorylation events and type 2 diabetes mellitus (T2DM). Detection of new phosphorylation sites (P-sites) is the first step toward better understanding of disease mechanisms and disease diagnosis. In this work we characterize the plasma phosphoproteome of Vietnamese T2DM patients using phosphoproteomics techniques.
Plasma proteins were digested with trypsin to produce tryptic peptides. Phosphopeptide enrichment was carried out using Immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO2) affinity chromatography. The phosphopeptide enriched samples were analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS). Phosphopeptide/phosphoprotein identification was carried out using the PEAKS software. Validation of peptide/protein identification was based on the Target-Decoy search strategy of which hits with FDR≤0.1% were selected for further analysis. Confident P-site localization was evaluated by the A-score algorithm. The identified phosphoproteins were functionally classified according to Gene Ontology Annotation using the STRAP software.
Phosphopeptides mapping phosphoproteins in human plasma were identified with FDR ≤ 0.1% and compared to PhosphositePlus. The biological analysis showed that, detected phosphoproteins showed different functions including: translation regulator activity, binding, receptor activity, structural molecule activity, catalytic activity, and transporter activity.
The present work introduced for the first time the plasma phosphoproteome of Vietnamese T2DM patients. The novel P-sites detected in the study could be the targets for subsequent studies on early diagnosis of T2DM.
Keywords: Phosphoproteomics, LC-MS, Vietnamese type 2 diabetes patients, human plasma

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Urine angiotensin II signature proteins as biomarkers of fibrosis in patients with kidney transplant

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Background

The activity of angiotensin-II (AngII), the main effector of the renin-angiotensin system (RAS), can lead to interstitial fibrosis/tubular atrophy (IFTA) in kidney allografts. However, there are no clinical markers of kidney AngII activity. We previously identified 83 AngII-regulated proteins in vitro, which reflected AngII activity in vivo. The urine excretion of 6 AngII-regulated proteins (BST1, GLUL, LAMB2, LYPLA1, RHOB and TSP1) was increased in chronic kidney disease patients. Here, we examined whether urine excretion of these 6 proteins reflected kidney IFTA and RAS activity in kidney allograft recipients.

Methods

Twenty patients from the Canadian National Transplant Research Program, with urine samples before and after RAS inhibition were selected. Another 19 patients with IFTA and 19 stable controls with concomitant urine and biopsy samples were selected. Urine excretion of 6 AngII-regulated proteins were quantified using selected reaction monitoring assays and adjusted by urine creatinine. Differences in urine excretion of AngII-regulated proteins between IFTA and control patients and patients on RAS blockade were assessed using t-test p-values with Benjamini-Hochberg adjustment. Rank's correlation was used to investigate correlations between AngII-regulated proteins and clinical parameters. Fixed effects correlation was used to assess changes in AngII-regulated proteins following RAS therapy.

Results

Urine excretion of AngII-regulated proteins was significantly higher in IFTA compared to control patients ($p<0.05$). These proteins also clearly separated IFTA and control patients in unsupervised clustering analysis. Urine excretion of all AngII-regulated proteins correlated with each other, but not with markers of kidney function, including serum creatinine and total urine protein. RAS inhibition decreased urine protein excretion of all AngII-regulated proteins.

Conclusions

We demonstrated an association between urine excretion of AngII-regulated proteins and IFTA. Urine excretion of these proteins was modified by RAS inhibition. These proteins may represent coveted markers of kidney fibrosis.

Keywords

Angiotensin II, RAS, fibrosis, SRM, biomarkers, kidney transplantation.

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Comparison of Pipelines for Differential Analysis of Label Free Quantification Experiments

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Background
Label Free Quantification by mass spectrometry is becoming a widely used technology to analyze the differential expression of proteins across multiple biological samples. To highlight those proteins which are differentially expressed, many questions are raised: how to filter, normalize, and impute the measured intensities? Which statistical test to use?
Herein, different pipelines for the differential analysis of protein intensities are compared. Our objective is to find pipelines allowing us to retrieve the maximum number of proteins known to be differentially abundant.

Methods
As a benchmark dataset, 48 human proteins were spiked into E.coli proteome at five concentrations. For each concentration, 4 technical replicates were injected on a Q-Exactive coupled to a nanoLC.
Raw data was analyzed with MaxQuant and Andromeda for feature extraction, peptide identification, and protein inference using the LFQ normalization algorithm. For statistical analysis, Perseus and R packages have been used to compare the impacts of the filtering, normalization, imputation and statistical tests onto the results. To assess their performance and to rank them, the rates of false negative and positive biological discoveries (FNR and FPR) were considered for each pipeline.

Results
From our results, it appears that data imputation and statistical testing influence the FNR and FPR the most. Data shows that the imputation methods like k-NN or MLE combined with appropriate filtering lead to lower FPR and FNR than other methods. Concerning statistical tests, empirical Bayes methods like LIMMA lead to better FNR with a similar FPR than traditional Welch and Student t-tests.

Conclusions
All pipelines lead to quite different lists of selected proteins and show that the differential analysis is crucial in the biological interpretation of results. Moreover, some of them seem to be more appropriate than others regarding the ranking established from the FPR and FNR on our data sets.

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Multiple myeloma associated bone marrow fibroblasts characterized by multi-omics analyses including proteomics, metabolomics and eicosadomics

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Background
Multiple myeloma (MM) is an incurable plasma cell tumor of the bone marrow. The disease is preceded by a non-malignant stage called monoclonal gammopathy of undetermined significance (MGUS), and, up until now, it is not well understood how the progression to the malignant stage takes place. However, there is some evidence that the tumor microenvironment, including tumor-associated stromal cells, plays a role in the pathogenesis of the disease. Aim of this study was to investigate more closely MGUS- and MM-associated stromal fibroblasts by multi-omics analyses.

Methods
Fibroblasts isolated from bone marrow aspirates of MGUS and MM-patients were subjected to a nano-LC-MS/MS analysis on a Q Exactive orbitrap mass spectrometer for proteome profiling, using the MaxQuant software for identification and label-free quantification of proteins. For comparison, normal human mesenchymal stem cells (hMSCs) were analyzed in parallel. Targeted metabolomics was performed by means of the AbsoluteIDQ®p180 Kit from Biocrates; eicosanoids were studied with an HPLC-coupled Q Exactive HF orbitrap mass spectrometer.

Results
From around 5500 detected proteins, more than 1000 were found to be significantly regulated between hMSCs, MM- and/or MGUS-associated fibroblasts with $p \leq 0.01$ and by applying multiparameter tests. While characteristic mesenchymal markers were present at comparable levels in all cells, important differences were observed for proteins related to hypoxia, redox regulation, apoptosis, cell cycle progression and different metabolic pathways. Results from proteomics, metabolomics and eicosadomics analyses gave indications how fibroblasts may promote MM progression and support tumor cell growth.

Conclusions
Fibroblasts in the bone marrow contribute to the establishment of a protective niche for myeloma cells. The integrative analysis based on multiple omics-disciplines allowed us to gain deeper insights into how these stromal cells may promote MM progression and support tumor cell growth.

Keywords
Clinical proteomics, multiple myeloma, multi-omics analyses

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Securitized production of stromal stem cells for therapy: Proteomic investigation on genetic stability and senescence

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Background
Mesenchymal stem cells (MSC) derived from bone marrow, as well as adipose derived stem cells (ASC) obtained from adipose tissues, can be used as reparative/regenerative cells to treat a range of clinical conditions. Because of the necessary small size of the donation, it is necessary to induce a large proliferation of these cells for clinical purposes, which may lead to senescence and increase the transformation risk. However, the changes actually induced by the culture process are still poorly understood.
Methods
We performed a large-scale proteomic characterization of MSCs and ASCs during cell culture, in order to identify and monitor protein markers or protein “signatures” that could guarantee the quality, genetic stability, and immunological efficiency of these cells. Cells from 3 different patients were cultured under hypoxia or normoxia conditions, and monitored at different stages of cell culture. Whole cell lysates, nuclear extracts, and secreted proteins were analyzed by nanoLC-MS/MS on a Q-exactive Orbitrap instrument, followed by label-free quantification. Dedicated MRM measurements were performed on low abundant proteins of interest.
Results
We obtained quantitative profiles during cell culture for several thousands of intracellular and secreted proteins of ASCs and MSCs, under normoxia or hypoxia conditions. While the cellular proteome appeared to remain globally quite stable, some proteins were found to vary significantly and reproducibly between early and latest stages of cell culture. Some variations were more pronounced in normoxia than in hypoxia, such as the increase of Cyclin-dependent kinase inhibitor 2A. Analysis of secreted proteins also allowed to point towards protein markers candidates that can be measured directly in the cell culture medium.
Conclusion
We generated an extensive proteomic dataset characterizing MSCs and ASCs in different conditions, and we propose a panel of candidate markers that may be used to monitor the stability of the cells during the culture process.

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In-depth proteome profiling of formalin-fixed paraffin-embedded urothelial carcinoma and benign disease tissues

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There are various benign proliferative conditions of the urinary bladder, but the more common ones are epithelial metaplasia, inverted papilloma, leucoplakia, nephrogenic adenoma, cystitis cystica, and cystitis gladulars. Among these, cystitis cystica (CC) and cystitis glandularis (CG) have been the most significant pathologically. These lesions are an unusual proliferative disorder of the urinary bladder, which is characterized by transitional cells that have undergone glandular metaplasia. Although cystoscopy and biopsy helps to confirm the diagnosis, no molecular markers for benign disease were discovered yet. Furthermore, information for molecular characterization of benign disease including CC and papilloma was limited so far. Here, we present comparison study of benign disease FFPE tissue specimens with urothelial carcinoma (UC) FFPE tissues using novel in-depth quantitative proteomic strategy. To perform quantitation in one FFPE slide per each samples, we developed well-defined proteomic strategies including FFPE protein extraction, filter-aided sample preparation, high-pH peptide fractionation based on stage-tip, and high-resolution quadruple Orbitrap LC-MS/MS. Label free quantification and data analysis were performed using Maxquant program. As a results, approximately 7000 protein groups were identified using one FFPE slide per sample, of which several hundred proteins were significantly regulated in between benign disease FFPE tissues and UC FFPE tissues. Some important proteins and novel proteins that related to pathology of benign disease and urothelial carcinoma were discovered using bioinformatics analysis and network analysis. Our in-depth quantitative FFPE tissue proteomic analysis platform will measure the levels of 7000 proteins with highly reproducibility from quite small amounts of individual human cancer or benign tissues. Our platform can easily be implemented in other type of cancer to analyze large numbers of pathologically relevant proteins in clinical specimens including FFPE tissues and biopsy samples.

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Platelet proteomics: towards non-invasive cancer detection

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Introduction
Platelets play an important role in tumor growth and, at the same time, platelet characteristics are affected by cancer presence. Therefore, we investigated whether the platelet proteome can be used as a source of biomarkers of cancer. To this end we performed several studies: study 1 (n= 8) and 2 (n=20) comprised platelet samples derived of patients with a variety of different tumor types and healthy controls; study 3 included platelet samples (n=25) of patients with pancreatic cancer (PDAC), chronic pancreatitis and controls, and study 4 comprised platelet samples (n=23) of patients with early stage lung cancer and pancreatic cancer and matched controls.

Methods
Blood was collected by free-flow after which platelets were isolated by several consecutive centrifugational steps, including washing steps. Label-free proteomics based on GeLC-MS/MS was used in conjunction with MaxQuant analysis for protein identification and quantification using spectral counting. The beta-binomial test was used to determine differential abundance.

Results
The platelet proteome datasets contained 2500- 4500 protein identifications. Differential analysis revealed that the platelet proteome of patients with (early-stage) cancer is altered as compared to that of controls. In addition, in study 4 we found that the platelet proteome normalized after tumor resection. Overall, upregulated proteins, subsets were primarily linked to inflammation, immune response and cytoskeleton organization and transport and downregulated proteins were involved in antigen processing and presentation among others. A subset of proteins more abundant in the cancer platelets proteome was found in more than one study.

Conclusion
This pioneering work on the platelet proteome in cancer patients clearly identifies platelets as a new source of protein biomarkers of cancer.

Keywords
Proteomics, platelets, biomarker, cancer

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Integration of "omics" strategies for the elucidation of molecular mechanisms underlying Brugada Syndrome

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The Brugada syndrome (BrS) is an inherited cardiac disorder responsible for 5-40% of sudden death. Given the high genetic and phenotypic heterogeneity of this disease, it has become increasingly clear that integrated approaches are needed in order to shed light on the complexity of the molecular mechanisms underlying the pathophysiology of BrS, as well as on the identification of reliable diagnostic/prognostic markers. In the current literature, a large number of studies is based on the use of individual classes of biomarkers; in our opinion, however, a synergic analysis of different categories of potentially interesting biomolecules might turn up to be more informative and useful.

For the present study, we selected a cohort of Brugada patients that were preliminary subjected to genomic analysis to assess the gene mutation involved in the pathogenesis of the syndrome. Plasma was obtained from each BrS subject and an integrated study of proteome and miRNome was performed to uncover novel diagnostic/prognostic markers and to shed further light on the molecular mechanisms underlying this complex and intriguing cardiac disorder.

Proteomic approach was based on the combination of proteominer-approach to reduce plasma sample complexity, with 2d PAGE, LC-MS/MS-analysis and western blot. miRNomic approach integrated the comprehensive profiling of 179 plasmatic miRNAs using the exiqon Serum/Plasma Focus microRNA qPCR plate whit targeted taqman assay for validation experiment.

IPA tool allowed us to combine "-omics" findings and to relate Brugada phenotype with peculiar deregulated pathways, suggesting a cooperative activity of mutated genes, deregulated miRNAs and proteins in orchestrating transcriptional and post-translational events that are determinant for the development of the Brugada cardiac phenotype. Our study provides the basis to deepen the role of specific molecular pattern on cardiovascular cellular signaling under homeostatic and pathological conditions and to gain an in-depth understanding of the pathogenesis and the progression of BrS.

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Targeting single amino acid variants in melanoma cell lines by parallel reaction monitoring

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Background
Single nucleotide variants (SNVs) are the most occurring variants in the human genome and highly accumulated in cancer cells. Many SNVs are non-synonymous (nsSNVs) leading to amino acid variants (SAVs) or protein truncations. We have recently showed that many SAVs affect modifiable amino acid residues in kinase target motifs of key signaling proteins and may alter their function in signal transduction networks. Variant peptides are challenging to detect and are mostly masked by biological background in routine MS analysis due to their low abundance indicating the necessity for targeted proteomic strategies.

Methods
We are currently developing a high resolution mass spectrometry (MS) approach to stratify and target variant peptides in a complex biological sample using parallel reaction monitoring (PRM) on the Q Exactive HF mass spectrometer. An in-house developed pipeline using RNA sequencing data enabled us to obtain information of theoretical SAV containing peptides in a melanoma cell line sensitive and/or resistant to a BRAF kinase inhibitor. Cells are lysed, proteins tryptic digested and peptide mixture is fractionated by high pH RP chromatography prior mass spectrometric analysis. Data is analyzed by MaxQuant and Skyline.

Results
Initial findings proof the presence of several variant peptides in targeted MS measurements. Among SAV-containing proteins we detected the adapter protein 14-3-3 eta involved in general and specialized cancer signaling pathways and Lamin B2, a potential chromatin interactor. A summary of our advancements towards the routine detection of SAV-peptides will be presented.

Conclusion
Targeted PRM analysis has a potential to detect and quantify individual SAV-containing peptides in a complex background and makes it amenable to future application in personalized proteomics.

Keywords
PRM, Targeted MS, Cancer

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Integrative analysis of transcriptomic and proteomic data from testicular tissues of Liver-X receptor knockout mice

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Background:Liver X receptors (LXRα/β) are members of the nuclear receptor superfamily. They act as ligand dependent transcription factors activated by oxysterols with crucial roles in regulating cellular cholesterol, fatty acid and carbohydrate metabolism. Both Lxrα/β isoforms are expressed in testis and important for male reproduction. By 6 months of age, the Lxrα/β double knockout male mouse (Lxrα/β DKO) develops sterility, low testosterone and aberrations in lipid homeostasis. The underlying mechanisms are not fully understood The aim of this study was to further our understanding of testis specific LXR functions and identify potential novel candidate genes/pathways deregulated in the testes of Lxrα/β DKO mice, using global expression studies.
Methods: Both RNA-seq and LC-MS analyses were used to study whole testicular tissues from Lxrα/β DKO mice in parallel with littermate age matched controls. cDNA libraries were prepared followed by next-generation sequencing (NextSeq 500). Extracted proteins were tryptically digested prior to LC-MS analysis. Peptides were separated using 1D nanoscale chromatography and data collected using an ion mobility assisted data independent (IM-DIA) mode of acquisition. Qualitative and quantitative data analyses were conducted using Progenesis QI for Proteomics, providing label-free quantitative results.
Results: Histological assessment of testicular tissues from Lxrα/β DKO mice revealed shrunken seminiferous tubules, cellular lipid droplet deposition and abnormalities in spermatogenesis. RNA-seq analysis identified 1185 differentially expressed genes ($p<0.01$) from Lxrα/β DKO versus control mice (log2 fold change -3.49 to +2.16). Quantitative mass spectrometry identified 647 differentially expressed proteins ($p<0.05$) (log2 fold change -9.32 to +4.02). Combined data sets show the top deregulated pathways in Lxrα/β DKO testes related to Eukaroyotic initiation factor 2 (EIF2) signalling, autophagy and protein ubiquitination.
Conclusion: LXRs may regulate EIF2 signalling which is crucial for successful spermatogenesis. Identification of biological pathways provides a greater insight into potential LXR functions and increase understanding of the reproductive defects.

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ENVIRONMENTAL ENRICHMENT INCREASES RIBOSOMAL, MICROTUBULE AND METABOLIC PROTEINS IN PIG HIPPOCAMPUS

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Environmental enrichment (EE) produces changes in brain structure and functionality. In rats and mice, EE enhances memory and learning abilities, it is associated to enlarged brain volume and it increases dendritic morphological complexity and neurogenesis, especially in the hippocampus. Pigs are an interesting animal model due to the resemblance of their brain to the human organ. In porcine production, EE is known to have positive effect on pig welfare.

In the present study, a total of 44 female pigs (crosses of Large White × Landrace NN sows with Pietrain heterozygous Nn boars) aged 8 weeks were housed in four pens of 11 animals each. From 8 to 15 weeks, pigs were allocated under the same housing conditions, which consisted in a full slatted floor with a density of 1.2 m2/pig. At 15 weeks, the density of two pens was reduced to 0.7 m2/pig with the same floor (barren) whereas on the other two pens the density was maintained, the floor changed to concrete with 700 g of straw/pig (enriched). At 24 weeks of age, pigs were stunned with CO2 and exsanguinated. The skull was opened, the brain was removed and the hippocampus (HC) was excised, rapidly collected in liquid N2 and kept frozen at -80 °C.

Proteins from the HC were extracted, labelled with iTRAQ and analyzed by nanoLC-ESI-MS/MS on an Orbitrap Fusion Lumos™ Tribrid (Thermo). The results indicate that 199 proteins were present in differential amounts ($p<0.05$), 114 upregulated and 85 downregulated (enriched/barren). The main three groups of upregulated proteins correspond to: 1) ribosome components and assembly (39 proteins), 2) tubulins (9 proteins) and 3) proteins related to oxidative phosphorylation (ubiquinone, succinate dehydrogenase, cytochromes, ATP synthase). This results are consistent with an increased hippocampal neurogenesis and higher metabolic activity of brain cellular components.

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Co-stimulatory Inhibition with Abatacept and the Effect on the Complement System in Rheumatoid Arthritis

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Background:
The increasing availability of biological disease-modifying anti-rheumatic drugs has tremendously benefitted rheumatoid arthritis (RA) patient life quality and mortality. A relatively new drug, abatacept, is a receptor-antibody fusion antibody that antagonizes the co-stimulation of antigen presenting cells on CD4+ T-cells. Abatacept has demonstrated therapeutic efficacy in RA which is an aggressive autoimmune related and inflammatory disease that actively degrades cartilage and eventually destroys the joints. There is still a need for development of new drugs, but the currently available drugs could be applied with more precision to decrease side effects. The aim of this study was to develop a proteomics based method for measuring abatacept in patients with RA and to investigate treatment effect on the complement system.
Methods:
We developed the targeted assay on pure drug samples, and spiked-in drug serum samples. 9 patients in stable abatacept treatment were enrolled into a time-series study of one week, i.e. one treatment interval. We applied DDA and PRM acquisition methods using a Q Exactive-HF on acquired serum samples from 6 time points during the treatment week.
Results:
A total of 169 serum proteins were identified at 1% false discovery rate from the relatively short gradient DDA runs and the complement system was analyzed in-depth. PRM was performed on five chosen abatacept peptides and the concentration of each peptide was calculated. For example, it was found that the compliment system responded to the drug concentration and then re-adjusted to near baseline values.
Conclusions:
Choosing optimal peptides from biological drugs can be challenging, but we developed the first targeted PRM assay on abatacept and analyzed the dynamically effects it exerts on the complement system. Based on the results, we propose that abatacept treatment somehow decreases the responsiveness to antibody-induced complement activation and we observe the between patient uptake to very individualized.

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Characterization of the signal transduction networks underlying the immunomodulation by the microbial metabolite trimethylamine.

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Background
Recently, the gut-brain axis has received significant attention and it has been shown that gut microbiota are able to modulate host inflammation. Evidence is accumulating that a microbial metabolite, trimethylamine (TMA), interferes with inflammatory response signaling pathways. Given the ever increasing importance of neuroinflammation in neurodevelopmental and neurodegenerative disorders, increasing our understanding of this modulation is vital.

Methods
In order to identify the phosphorylation events underlying anti-inflammatory effects of TMA, two triple SILAC experiments using a common control were used to perform a quantitative phosphoproteomic screen of BV-2 microglial cell cultures induced by 20 ng/ml LPS in combination with 200 µM or 600 µM TMA in two biological replicates. Doses and treatment durations were established using ELISA assays prior to MS measurements. After tryptic digestion, peptide samples were split for both proteome analysis and TiO_2-based phosphopeptide enrichment of each triple SILAC experiment. Proteome and phosphoproteome were analyzed using an EASY-nLC 1200 coupled to a Q-exactive HF mass spectrometer. Data processing and analysis was performed using MaxQuant, Perseus and IPA software packages.

Results
In preliminary measurements we quantified 5,364 proteins and 7,050 phosphorylation sites. Biological replicates showed good reproducibility at the proteome and phosphoproteome levels. Using SILAC-labelled BV-2 microglial cells, we showed that TMA is able to modulate LPS-induced IL-6 secretion. Gene Ontology analysis of differentially expressed proteins showed that TMA reduces many specific LPS-induced inflammatory functions, and specifically enriches for differentially expressed mitochondrial and ubiquitinating proteins. Comparison of 200 µM and 600 µM TMA treated cells showed a dose dependent modulation of inflammatory signaling.

Conclusions
TMA effectively modulates the secretion of IL-6 in LPS-challenged microglial cells. Furthermore, functional analysis of the proteomic data shows that TMA effectively ameliorates many aspects of the molecular signature indicative of inflammatory activation during LPS challenge.
Keywords
SILAC, phospho-proteomics, neuro-inflammation, neurodevelopmental disorders, trimethylamine

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Quantitative assessment of the phosphoproteomes of kinase-deficient Escherichia coli strains

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Background

Protein phosphorylation on serine, threonine and tyrosine (S/T/Y) serves as a fundamental regulatory mechanism in nearly all physiological processes in bacteria, particularly in the infection process. e.g. cell adhesion, perturbation of host signaling cascades and impairment of defense mechanisms. Dysregulation of phosphorylation is often linked to pathogenicity, thus phosphorylation mechanisms are expected to be promising targets for new drugs able to overcome antibiotic resistance. Phosphoproteomic analysis has emerged as a promising tool for studying variations in protein phosphorylation in eukaryotic cells, however the much lower degree of protein phosphorylation in bacteria (about 80 times less) has severely hampered progress. The extent of phosphorylation events and the kinases associated with phosphorylation sites remain unknown.

Methods

To decipher the kinase-target interactions, we constructed kinase-deficient E. coli K12 strains (ΔaceK and ΔyeaG) and characterized their growth phenotypes (malate and acetate) and phosphoproteomes.

Preliminary results

SILAC-based quantitative phosphoproteomic analysis enabled identification of potential substrate proteins for each kinases. Preliminary data show striking differences in classes of phosphorylated proteins and distribution of S/T/Y phosphorylation sites when grown on malate or acetate. For functional follow-up studies, we have initiated cloning and expression of aceK and yeaG. In vitro phosphorylation assays will be performed to confirm the detected substrate proteins.

Conclusions

The generated data can be used to identify roles of the different kinases and profile the S/T/Y signal transduction network in E. coli.

Keywords

Kinases, bacterial proteomics, phosphoproteomics.

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Enhanced proteome coverage of MCF7 cells using an integrative search engine approach

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Background
The accurate and unambiguous detection of all human proteins using mass spectrometry-based technologies is one of the Human Proteome Project (HPP) main goals. Specifically, HPP initiative develops new methodological approaches to characterize missing proteins, namely those without MS experimental evidence in the neXtProt database. In order to select the biological samples where these proteins are expressed, bioinformatic pipelines have been developed to scrutinize publicly available transcriptomic and proteomic experiments.

Methods
We propose a new method for the analysis of high throughput proteomic experiments to increase the proteome coverage. The integration of peptide identifications from 4 search engines (MASCOT, X!Tandem, OMSSA and Comet) contributes to the detection of missing proteins. We tested our bioinformatics pipeline with the human breast adenocarcinoma cell line MCF7 from the public NCI60 proteomics dataset. We used the same parameters for all search engines and the results were compared following a HPP guidelines compliant procedure.

Results
The analysis of MCF7 datasets revealed a high degree of coincidence among the identified proteins with either search engine. However, although a high percent of the proteins were common identifications, a significant number of search engine specific assignments were also observed. This approach allowed us to find 18 missing proteins.

Conclusions
We developed a bioinformatics pipeline based on the integration of different search engine identifications. This approach enables the re-analysis of public datasets to increase the coverage of the human proteome in the context of the HPP. We analyzed the MCF7 cell line proteome as a proof of concept to test the viability of this method and we concluded that the proper combination of search engines improves the detection of missing proteins in publicly available experiments.

Keywords
C-HPP, missing proteins, NCI60, integration of search engines

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Maximizing proteome coverage on an Orbitrap Fusion Lumos with an advanced precursor determination algorithm

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Background: Modern hybrid mass spectrometers are incredibly versatile instruments that combine multiple MS technologies into a single platform. During data-dependent LC-MS/MS, these technologies work together to sequence tens of thousands of peptides in complex proteomic samples. To push sampling depths even further requires that every component in the hybrid instrument is operating at optimal efficiency. To this end, we have developed a new Orbitrap precursor determination algorithm that identifies hundreds of thousands of additional precursors in FTMS1 spectra. To facilitate better sampling of all these additional precursors, we configured the quadrupole ion trap to collect MS2 spectra at a rate of 50 Hz.

Methods: We analyzed human proteome samples with a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer coupled to a Thermo Scientific Easy-nLC 1000 ultra-high pressure LC.

Results: The Orbitrap Fusion Lumos mass spectrometer typically collects ~100,000 MS2 spectra during a standard two hour data-dependent LC-MS/MS analysis of a complex proteomic sample. This relatively large pool of MS2 spectra converts to ~25,000 unique peptide identifications or ~5,000 proteins. Though this level of proteome coverage is already quite extensive, off-line analysis of the same LC-MS/MS data set reveals that there are hundreds of thousands of unique LC-MS features that were never interrogated by MS/MS. Working together, our advanced precursor determination algorithm, and our optimized method for fast ITMS2 spectral acquisition, thoroughly interrogate all the available precursors in the FTMS1 spectra. More than 200,000 MS2 spectra are collected with this optimized method during the same two hour LC-MS period. This translates into 50% more PSMs, 30% more unique peptide sequences, and 10% more proteins.

Conclusions: We dramatically improve proteome coverage during data-dependent LC-MS/MS with a more sensitive precursor determination algorithm and an ITMS2 method capable of very fast ITMS2 spectral acquisition.

Keywords: Tribrid MS, spectrum processing, ITMS2

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Quantitative mass spectrometry to identify oviductal fluid proteins related to reproduction

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Background
Body fluids are a unique source of molecules in the study of cell communication and distinct proteins might directly reflect intracellular events. Fluids from the female reproductive tract are key to reproductive processes. Amongst others, the oviductal fluid (OF) serves as a host for gametes. The molecular composition is dynamic and adapted to the respective biological events. Herein, we aimed to identify candidate proteins from the OF that are involved in the events prior to fertilization.
Methods
OF samples were collected by flushing different segments (ampulla, isthmus) of explanted rabbit oviducts at different time points after insemination (4-8h). Proteins were fractionated using a ConA/WGA lectin based approach. Relative quantification between treated and control samples was performed by dimethyl labeling and analysis by a TripleTOF® 5600 MS system. Raw data were processed using MaxQuant and a custom R based analysis pipeline.
Results
In total 421 unique proteins were quantified, of which 109 were differentially abundant. 116 enriched glycoproteins were identified in ampullary OF samples. In silico analysis highlighted that differentially abundant proteins were significantly enriched in the GO terms “response to stress”, “protein activation cascade”, and “regulation of response to external stimulus”. Comparative analysis revealed differences in the OF composition related to the functional segments. The OF from the isthmus in contrast to the ampulla segment showed a significantly different abundance of glycan binding proteins (galectins) and apolipoproteins. Selected proteins were validated by antibody based assays.
Conclusion
These findings demonstrate that the OF is significantly changed early after insemination. Differentially regulated proteins like the galectin LGALS3 and Toll-like receptor co-chaperone CNPY3 suggest a role of the immune system within the oviduct in events preceding fertilization. Taken together, we provide a unique insight into the regulative activities occurring in the OF upon insemination.
Keywords
Oviductal fluid, glycoproteins, fertilization, dimethyl labeling, lectin

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Making Annotated Spectra Available when Standard Formats are not an Option

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Background
The sharing of proteomic data was initially driven by the recognition by journals that authors cannot look at all of their reported results, so it is desirable to make available data associated with a manuscript to allow independent assessment and reanalysis of results of particular interest. This process has been heavily facilitated by the development of standard formats by the HUPO PSI. However, for some search engines, outputs in these formats are not an option, presenting a challenge for authors to comply with journal publication guidelines.

Methods
MS-Viewer, part of the free Protein Prospector package, can produce annotated spectra from many search engine outputs, including simple tab-delimited text files. This allows it to annotate data from search engines that do not support standard HUPO-PSI formats. It can also annotate cross-linked peptide identifications. A website allows submission of results to a public repository (msviewer.ucsf.edu), and there is a searchable browser that allows one to see all submissions associated with publications.

Results
MS-Viewer has supported about twenty publications in the last year and is also used for sharing results between laboratories. It is particularly popular with MaxQuant users, as it allows production of annotated spectra from much smaller file uploads than through using the MaxQuant Viewer and can handle large datasets that the MaxQuant Viewer struggles to display. Working with authors, we have successfully produced annotated spectra from all submissions.

Conclusions
While conversion of results into a standard format is most useful for data re-use, MS-Viewer allows all researchers to make annotated spectra available and get their data published. It allows re-sorting and searching of data to easily allow people to find data of interest.

Keywords
Search Engine Results; Annotated Spectra; MaxQuant

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Protein modification and the immune system; a ménage a trois gone wrong in Multiple Sclerosis.

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Background
Peroxidation products have been suggested as biomarkers for inflammation in numerous diseases, but their role in the etiology is not fully understood. In the autoimmune disease Multiple Sclerosis(MS) a significant upregulation of some of these peroxidation derived products have been reported both in the cerebrospinal fluid and serum samples, and suggested as points to monitor pathogenic mechanisms of the disease. Resent research in Rheumatoid Arthritis has suggested peroxidation derived protein adducts as key factors in breakage of tolerance to the native protein in a process called altered peptide ligand response, thus generating the autoimmune response. These adducts could be highly relevant in etiology of the autoimmune response towards key proteins in the myelin sheet in MS, and potentially be the origin of some of the initial epitopes for T and B cells.
Methods
Using Mass Spectrometry based approaches, we seek to investigate more of the etiology behind MS focusing on the role of peroxidation, the interaction between myelin basic protein (MBP) and lipids as well as novel protein modifications potential effect on the formation of complexes between MBP and lipids or adduct hereof.
Results
A novel hypothesis considering the identification of lipids which covalently bind to MBP has been tested and confirmed using Mass Spectrometry. We have focused on epitope generating lipid derived adducts as well as other protein modifications like citrullination, and suggested a revised disease induction and progression model involving these different findings.
Conclusions
Expansion of knowledge in metabolism interaction with inflammation focusing on protein and lipid modifications, as well as crosstalk in MS will contribute to the understanding of the disease etiology. Potentially, this could provide retrospective information leading to diagnostic as well as prognostic biomarker discovery and thereby to potential target molecules for novel drug discovery.
Keywords.
Lipidation, Post-translational modifications, Multiple Sclerosis, Myelin Basic Protein.

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Proteomics investigation of Wilson's disease pathophysiology in the ATP7B-/- murine model

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Background
Wilson's disease is a rare genetic disorder triggered by mutations in the ATP7B gene. These mutations lead to the dysfunction of a transport protein (ATP7B) involved in copper excretion. This disease is characterized by defects in copper transport and by toxic copper overloads, primarily in the liver and the brain. Copper toxicity leads to liver injury and neuropsychiatric disorders which manifest either as acute (fulminant hepatitis) or chronic (cirrhosis) episodes. In this study, we explore the plasma and liver proteome modifications induced by disease development in the ATP7B-/-murine model for Wilson's disease.
Methods
The ATP7B-/-mouse was used as a preclinical model of Wilson's disease. Plasma and liver samples from ATP7B-/- and wildtype mice were collected at defined stages of the disease. Plasma and liver proteomes were characterized and compared using either a SUPER-SILAC or a label-free quantification strategy. Data analysis was performed using MaxQuant and Prostar softwares. Proteins identified as differentially abundant between ATP7B-/- and wildtype mice were quantified in a larger group of samples using LC-SRM and AQUA peptides. Skyline software was used for LC-SRM data analysis.
Results
Proteomics discovery investigations using ATP7B-/- and wildtype mice at different stages of Wilson's disease led to the identification of 31 plasma proteins specifically regulated during disease progression. Over this panel of proteins, 5 proteins were validated by quantitative LC- SRM in a larger group of plasma samples. In the liver, proteins involved in metal homeostasis, lipid metabolism, carbohydrate and energy metabolism were found to be regulated during Wilson's disease development.
Conclusions
Using discovery and targeted proteomics analyses in a mouse model of Wilson's disease, we could identify potential plasma biomarkers for disease progression. In the liver, we identified proteins that may play a major role in copper buffering during disease progression.
Keywords
Wilson's disease, Proteomics, Biomarkers, Mass spectrometry

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Adipocyte exosomes promote melanoma aggressiveness through Fatty Acid Oxidation: novel mechanism linking obesity and cancer

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Background: Recent results have demonstrated that mature adipocytes, the main cellular components of adipose tissue, favor tumor progression by secreting proteases and pro-inflammatory cytokines. Understanding the role of adipocytes in cancer is of major clinical importance as it is now largely acknowledged that obesity, in which the normal balance of adipose tissue secretions is modified, affects cancer incidence and prognosis. Recent evidence demonstrates that exosomes play a pivotal role in local and systemic cell-cell communication in cancer. Very little data are available concerning the composition and function of exosomes-secreted by adipocytes, and their role in cancer progression has never been investigated.
Methods: Adipocyte-derived exosomes were purified and their protein content was analysed by mass spectrometry. NanoLC-MS/MS coupled with LTQ-Orbitrap Velos mass spectrometer was used, followed by Mascot Daemon bio-informatics analyses.
Results: Among the proteins identified, all common exosomal markers were present. Interestingly, these exosomes were found to carry many proteins implicated in fatty acid β-oxidation (FAO), a feature that is highly specific to adipocyte-derived exosomes. Inhibition of this metabolic pathway in tumor cells completely abrogates the increase in migration/invasion observed in the presence of adipocyte-derived exosomes. Moreover, in the presence of adipocyte exosomes, FAO rates are increased in melanoma cells. In obese individuals, both the number of exosomes secreted by adipocytes as well as their effect on FAO-dependent cell migration are amplified.
Conclusions: This study characterizes the proteome content of adipocyte-derived exosome and shows,for the first time, that these exosomes can favor cancer progression. Finally, obesity profoundly influences tumor progression by modifying the number and the content of the exosomes secreted by adipocytes.
Keywords (5 max): exosome, adipocyte, nanoLC-MS/MS, cancer

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Dissecting the TRIM8 role in the pathogenesis of glioblastoma

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Background
Tripartite motif (TRIM) proteins constitute a superfamily that share a conserved motif architecture known as RBCC. Some TRIM proteins possess E3-ubiquitin ligase activity and are involved in many biological processes and changes in their abundance or activity are associated with several pathological conditions. In particular, TRIM8 controls multiple physiological functions, including inflammation, cell survival, differentiation and exerts central roles in cancer. TRIM8 is aberrantly expressed in glioblastoma, a lethal brain tumor that arises from glial cells, and its expression inversely correlates with tumor grade. In order to find novel approaches for glioblastoma treatment, molecular mechanisms responsible of the glioma onset and involving TRIM8 were investigated by a functional proteomic approach.
Methods
Immunoprecipitation experiments and LC/MSMS analyses were performed on mouse embryonic neurospheres cells expressing FLAG-Trim8 in order to purify and identify protein complexes involving FLAG-Trim8. Cells expressing an empty vector were used as negative control. Co-immunoprecipitation experiments were carried out and interaction analysed by western blotting.
Results
Almost 50 TRIM8 putative interacting proteins were identified and clustered in biological processes and pathways according to UniProt annotation. Eight mitosis-related protein (22%) were identified; four of them are members of the kinesin-like protein family. Co-immunoprecipitation assays showed that Trim8 physically bound to previously identified Kif11, Kif2c, Kifc1, and Haus. Hence, functional relationships between Trim8 and mitotic motor proteins were also explored.
Conclusions
Our data show that TRIM8 is involved in regulation of mitotic spindle machinery: during mitosis, different dynamic morphological transitions are coordinated in a temporal and spatial manner through ubiquitination of key mitotic factors, providing directionality and fidelity to this process. TRIM8 aberrations lead to mitotic checkpoint defects, common also in human glioma cancers.
Keywords
TRIM8, glioblastoma, mitosis, kinesins, ubiquitination

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MAPPs (MHC-II Associated Peptides ProteomicS): an in-vitro, pre-clinical tool to compare candidate compounds' immunogenic liabilities

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Background

Immunogenicity of biopharmaceuticals often leads to loss of efficacy and may cause severe side effects due to the formation of anti-drug antibodies (ADA). MAPPs is a mass spectrometry-based methodology to identify and relatively quantitate peptides processed by dendritic cells and presented through MHC class II receptors that may recruit and activate T cells to trigger the immunogenicity cascade.

Methods

Monocytes are isolated from healthy donors' peripheral blood mononuclear cells (PBMC) and differentiated into dendritic cells, which are then loaded with the compounds of interest. Cells are lysed and the MHC-II/peptide complexes are immunoprecipitated. MHC-II peptides are analyzed by nano-flow liquid chromatography tandem mass spectrometry, after which raw data are processed and normalized using a customized in-house designed workflow. Results are visualized in Spotfire.

Results

We present here our latest development in establishing the MAPPs workflow to assess potentially immunogenic biopharmaceuticals. The commercially available monoclonal antibody Remicade (Infliximab; 18-25% clinical ADA incidence in healthy volunteers*) was investigated in this study to demonstrate the repeatability and robustness of the assay. We further demonstrate the analytical power of MAPPs in the analysis of SS1P (a highly immunogenic anti-mesothelin immunotoxin) and an aggregated monoclonal IgG1 model antibody in the identification of potential T cell epitopes.

Conclusions

The present assay format enables a robust head-to-head comparison of up to 14 conditions (titration of one compound and/or comparison of compounds including positive/negative controls) per donor, demonstrating its applicability in comparing candidates' compounds immunogenic liabilities prior to clinical lead selection.

Keywords

MAPPs, immunogenicity, MHC-II peptides, anti-drug antibodies

*: Lambert J, Wyand M, Lassen C, Shneyer L, Thomson E, Knight A, Willers J, Kay J. (2016) Bioavailability, safety and immunogenicity of biosimilar infliximab (BOW015) compared to reference infliximab. Int. J. Clin. Pharmacol. Ther. 54(4) 315-322.

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RiboZinB: Identifying the actively translated isoform. The case of the Zero-inflated model.

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Background
Ribosome profiling (ribo-seq) is a promising new technique for characterizing the occupancy of ribosomes on messenger RNA (mRNA) at base-pair resolution. Ribosomes translate mRNA into proteins so information about its location on the genome can be used to deduce the actively translated isoform(s) in a phenotype. Most eukaryotic genes encode multiple splice-isoforms but only one or a few are (co-)expressed. A key challenge in to determine the isoform(s) actively translated as the short reads often map to multiple isoforms of the same gene.

Methods
In this study, we present RiboZinB a novel data-driven statistical model to identify the actively translated isoform(s) in a sample under investigation. We hypothesize that the distribution of the ribosome protected fragments (RPF) reads across a gene are representative of the isoform(s) actively translated in the sample. We compare the RPF read distribution of each isoform to its corresponding gene and define a score to quantify the similarity between the two distributions. Based on the binomial distribution pattern of RPF reads on an mRNA and the fact that many of the mRNA positions had zero reads, a zero-inflated negative binomial model was established for the gene-to-isoform comparison.

Results.
We applied the RiboZinB model onto two in-house ribo-seq data from human HCT116 and mouse embryonic stem cells (mESC) to identify the expressed isoform(s). We observed that the canonical isoform is not the most prominent isoform in about 9 and 10% of the expressed genes in the human HCT116 and mESC data respectively.

Conclusion.
The ability to identify the isoform(s) actively translated within a phenotype can improve our understanding of translation regulation between phenotypes as well as provide a more comprehensive search space to aid in mass spectrometry based protein identification. RiboZinB provides a means to assess the proportion of alternative spliced isoforms that are actively translated.

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Characterization of protein-protein interactions in the synapse by chemical cross-linking mass spectrometry

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Background
Synapses are essential in neuronal function to transmit signals to target cells. Thereby, a complex protein interaction network fulfils numerous tasks like synaptic vesicle docking, fusion and recycling. We set out to analyse synaptic protein-protein interactions by cross-linking mass spectrometry (XL-MS). So far, XL-MS has mainly been used to characterise samples of low complexity. However, analysis of cross-linked peptides is challenging when applied to complex systems like organelles and cells. Recently, several computational and chemical strategies have been introduced that allow the expansion of XL-MS to a quantitative and global scale. We demonstrate how different cross-linking agents and an optimized database search can be used to identify synaptic protein-protein interactions.

Methods
Purified rat brain synaptosomes were cross-linked with non-cleavable as well as MS- and thiol-cleavable cross-linkers. Cross-linked peptides were enriched by SCX and peptide SEC and measured on Orbitrap Tribrid mass spectrometers. Peptides cross-linked with MS-cleavable cross-linker were analysed using XlinkX, whereas a restricted database was created by conventional database search with Maxquant from cleaved linear peptides initially cross-linked with thiol-cleavable reagents. Non-cleavable, isotopically labelled cross-linkers were used under different conditions to detect changes in protein-protein interactions.

Results
We compared cross-link identifications between all three cross-linking agents and search strategies. Compared to the use of a top300 database of most abundant proteins, the restricted database resulted in 53 % more identifications and a reduced search space while including almost 3000 proteins. The use of an MS-cleavable cross-linker supported database search against the entire rat proteome, but led to less identifications. Identified cross-links included core components of the synaptic fusion machinery.

Conclusions
The restricted database workflow is a valuable alternative for the analysis of complex samples omitting the focus on most abundant proteins. It is further comparable to MS-cleavable cross-linking strategies.

Keywords
Cross-linking, database restriction, synaptosome, cleavable cross-linkers

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Multiplexed Mass Spectrometric Screening of EGFR Mutation in Non-small-cell Lung Cancer

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Background
High throughput sequencing of cancer genome has accelerated the comprehensive characterization of somatic mutations. However, the expression levels of mutation bearing genes and their translation protein variants are not always quantitatively correlated. Thus, we aim to develop a MS-based onco-proteogenomics platform which integrates cancer proteomics and genomics for multiplexed screening of tumor-specific mutations on oncoproteins. The well-known EGFR mutations, which have been discovered associating with response to EGFR trypsin kinase inhibitors in lung adenocarcinoma patients, were used as a model.
Methods
The non-small cell lung cancer cell lines harboring different EGFR subtypes were used in this study. We applied in-silico digestion for the 40 EGFR mutations with different proteases to generate potentially detectable EGFR mutated peptides and select adequate enzymes for comprehensive detection. Based on the in-silico digestion, we developed oncoproteogenomics strategies integrating affinity purification of EGFR protein complex, parallel enzymatic gel-assisted digestions, LC-MS/MS analysis and customized database searching using multiple engines, for identification of 34 mutated versus 33 wild-type EGFR proteins. All PSM and peptide matches were filtered to 1% false discovery rate.
Results
In the analysis of PC9 cells, EGFR del_746-750 peptide was identified with high confidence by the developed oncoproteogenomics strategies, as evidenced by fragment ions covering the deletion of 5 amino acids (746-750_ELREA) in the mass spectrum. In addition, the corresponding wild-type peptide was identified in different enzymatically digested sample. A series of signature fragment ions representing LREA resides were confidently assigned to confirm the sequence of wild-type peptides. The identification of paired EGFR L858R and wild-type peptides were also confidently obtained in heterozygous EGFR_L858R/WT H3255 cells.
Conclusion
These results indicated that our proposed MS-based oncoproteogenomics strategies can precisely determine the status of somatic mutations at protein level. Quantitation of the wild type and mutant forms is ongoing.
Keywords
Lung cancer, oncoproteogenomics, mass spectrometry, EGFR

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Novel permutation scheme improves accuracy of permutation-based false discovery rate estimation in quantitative proteomics.

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Background
Finding proteins whose expression levels are significantly changing upon a stimulus has its challenges due to often small replicate group sizes used in proteomics and the simultaneous testing of multiple hypotheses. A popular way of dealing with this is to control the false discovery rate with permutation-based methods. Usually, for generating the background distribution one uses all permutations of the (treatment/control) grouping or fuzzy modification of the technique [1]. Here we show that this standard method of generating the background distribution leads to a biased estimate of the proportion of false positives. We suggest a novel method which on controlled benchmark datasets yields a correct, unbiased estimate.
Method
We implemented a new method for subsampling the space of all permutations with the goal of making the background groupings 'orthogonal' to the original grouping. We extend the new method to multiple groups (ANOVA) and unequal group sizes. Finally, we performed an extensive comparative analysis of multiple permutation as well as standard FDR control methods using synthetic (normal distribution with variable mean value) and real experimental data (the mixture of HeLa and E. coli samples).
Results
Comparative analysis based on synthetic dataset shows that on average the 'orthogonal' permutation method better approximates the real false discovery proportion, especially for the case of small group sizes. A similar trend is observed in the experimental data. This and all mentioned methods are accessible in the Perseus software platform.
Conclusions
Here we introduced the new 'orthogonal' permutation method for the FDR estimation. Based on the numerous benchmark datasets we conclude that new 'orthogonal' method will benefit in a case of small replicate group sizes which is often the case in proteomics studies.
Keyword
FDR, statistics, Perseus, multiple hypothesis testing

[1] Yang et al, Scientific Reports 6 (2016).

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Investigations of LysN cleavage capability at biotinylated lysines

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Background
The metalloendopeptidase LysN has been described as an alternative protease in LC-MS based proteomic approaches. Compared to trypsin, LysN generates larger peptides containing an amino-terminal lysine which directly affects protein sequence coverage and improves ETD-based fragmentation. It has previously been shown that LysN is able to cleave N-terminal of mono- and dimethylated lysines. Here we describe the initial experiments for verifying the cleavage ability of LysN at biotinylated lysines. Together with the ionization and fragmentation properties of LysN derived peptides, such a proteolytic capability would enhance many biotin-based proteomic approaches such as membrane protein mapping.

Methods
Two different synthetic peptides were biotinylated by NHS-Biotin, incubated with LysN and analyzed by MALDI-TOF. The corresponding MS spectra were compared to those of non-biotinylated, LysN digested controls in order to identify impaired cleavage behavior.

Results
MALDI-TOF analyses indicate the ability of LysN to cleave at the N-terminus of biotinylated lysines. Compared to the non-biotinylated control, the cleavage ability of LysN amino-terminal of biotinylated lysine was strongly reduced at the experimental conditions tested. However, prolongation of the LysN incubation time seemed to improve proteolytic activity.

Conclusions
The preliminary results suggest the ability of LysN to cleave N-terminal of biotinylated lysines. This introduces a promising addition to the proteomics toolbox in a large variety of methods e.g. in protein surface mapping and PTM analyses.

Keywords
proteomics – membrane proteins – proteolysis – PTM – biotinylation

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Proteomics Analysis of White Blood Cells in Healthy Humans

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Introduction:
Proteomic analysis of peripheral blood mononuclear cells (PBMC) can be used to characterize age-related changes in immune function and may explain the origin of the pro-inflammatory state with aging. In this study, we characterized the proteomes of whole PBMC and 11 cell populations from 25 healthy human donors ranging in age from 20 to 82 years and separated into 5 age groups (20–34; 35–49; 50–64; 65–79 and 80+years). Thus, changes that occur with aging across different cell types may then be interpreted as an immunological aging "signature."
Methods:
PBMC samples were FACS sorted into "pure" cell lines and prepared for proteomics by standard methods. Peptides from each cell population were analyzed in a TMT labelled LC-MS/MS experiment on an Orbitrap. The data was searched and combined by prophet algorithms, normalized and validated on Scaffold with 0.1% peptide FDR. A linear mixed regression model was implemented to examine age effects.
Results:
In this pilot proteomics study, B cells (memory and naive), T cells (CD4+ and CD8+, effector and central memory, and naive) natural killers, monocytes, granulocytes and whole PBMC were analyzed. We found that numerous proteins changed with aging in all 11 cell types. Particularly strong age-effect were detected for B memory cells, CD8+ central and effector memory T cells, which is interesting, as these are long-lived cells, and expected to show an aging phenotype. Our findings suggests that memory cells may be a driver of inflammation-associated physiological changes in aging, and individual subject variation in these changes may be due to early immunological exposures.
Conclusion:
This preliminary result establishes an analysis pipeline for characterization of the aging-related immunological proteome, and reports preliminary proteomic results.
Keywords:
Aging proteome, Immunosenescence, WBC, PBMC, Proteomics

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Proteomics Analysis of Skeletal Muscle in Healthy Human

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Introduction:
In recent years, the human proteome has been studied across human cell types and tissues, producing draft maps of the human proteome. However, to date, no study has explored how the human proteome changes with aging in specific tissues. In this study, we have examined the proteome of healthy human skeletal muscle utilizing a TMT-based protein quantification approach.
Methods: The skeletal muscle samples were collected from 25 healthy human donors ranging in age from 20 to 82 years and separated into 5 age groups (20–34; 35–49; 50–64; 65–79 and 80+ years). Tandem Mass Tag (TMT) 6+ was used for relative protein quantification. Skeletal muscle samples were extracted, trypsin digested, reduced and alkylated. MS/MS peaks from the samples were searched with Mascot and X!Tandem. The two searches were combined by PeptideProphet and ProteinProphet on the Scaffold analysis system. For final representation, spectra were normalized by median polish and median sweep, protein identifications were quantified, and annotated.
Results:
Relationships of different proteins with aging were examined by linear regression models. Several functional classes of proteins were found to be altered during aging, including oxidoreductase, cytoskeletal, chaperones, isomerase and peroxidase proteins. Changes with aging of TCA cycle, respiratory chain and the electron transport pathway, ATP synthesis and branched chain amino acid catabolism pathway were evident. Our preliminary analysis suggests that insulin receptor signaling, eNOS signaling, TNF-beta, and IL-6 are up-regulated with age and apoptosis and mTOR signaling are down-regulated with aging.
Conclusion:
Our preliminary analysis shows that the skeletal muscle proteome undergo substantial changes with healthy aging, indicating profound changes in energy metabolism.
Keywords:
Aging, Skeletal Muscle, Proteomics, Energy Metabolism

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Studying mechanisms using omics-based approaches in ischemia reperfusion injury associated with kidney transplant

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Background: Kidney transplantation is the optimal treatment for end-stage kidney disease, as it prolongs life, improves the quality of life and results in cost savings, compared to staying on dialysis. However, the continuous organ shortage remains a serious problem leading to the use of marginal grafts. These grafts tolerate cold storage poorly, resulting in more severe ischemia-reperfusion injury (IRI) and a higher rate of delayed graft function (DGF), defined as the need for dialysis in the first week after transplant. The increased usage of marginal grafts has led to the development of alternative preservation techniques to reduce the IRI. Normothermic ex vivo kidney perfusion (NEVKP) is a storage method that results in improved kidney function compared to traditional static-cold-storage (SCS) in a porcine model of IRI and kidney transplantation. We sought to determine the molecular mechanisms behind the beneficial effects of NEVKP.

Methods: Kidneys from pigs were explanted, subjected to 30-minute warm ischemia time, and then placed in SCS or NEVKP for 8 hours, followed by reimplantation into the pig. Kidney biopsies were taken at time of explant, 30 minutes following anastomosis and on day 3 post-transplant from 5 pigs in NEVKP group and 5 in SCS group. Biopsy tissue was subjected to proteomic analysis on Q-Exactive mass spectrometer.

Results: 6179 proteins in total were quantified (FDR 0.01). Ninety-two and 249 proteins were differentially expressed ($p<0.05$; paired t-test) between NEVKP and SCS groups at 30 minutes and on day 3 post-transplant respectively, with top enriched pathways being cellular metabolism, catabolism, oxidative stress and translation ($p<0.005$; Cytoscape pathway enrichment analysis).

Conclusion: The identification of key proteins and mechanisms involved in IRI may assist in selecting donor organs and lead to testing of novel compounds that may enable improved graft preservation and diminished IRI.

Key Words: Ischemia-reperfusion injury, transplantation, proteomics, kidney

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A method for selecting a representative protein of the highest protein evidence from non-unique proteins.

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Background

The C-HPP Data Interpretation Guidelines require large-scale results thresholded at < 1% protein-level global FDR. Open-source Fido and Mayu software can be used for estimating FDRs at the protein levels, but their results contained many lower protein-evidence (PE) proteins with redundant peptides, which hinders inference of proteins with the highest protein evidence. We developed a simple SQL code that replaces these non-unique proteins with a representative protein with the highest protein evidence.

Methods

Data for laser-microdissedted samples of the lepidic-type lung cancer groups（AIS, MIA, LPIA in increasing invasiveness order) were searched for Uniprot human fasta by crux tide-search with exact p-value calculation, the results were validated with percolator (q-value<0.01), and protein-level q-values were calculated with Fido. Non-unique proteins were replaced by representative proteins with SQL codes using the following order of priority: 1, A protein containing a unique peptide with the highest protein evidence and the alphanumerically first entry name; 2. A reviewed protein with the highest protein evidence and the alphanumerically first entry name; 3, un-reviewed proteins with the highest protein evidence and the longest amino acids.

Results

There is a total of 788 proteins identified for lung cancer groups, increasing to 2305 by the addition of frozen normal tissue data at the threshold protein level FDR <0.01. Selecting the highest PE proteins with this method lead to the enrichment of reviewed proteins in the final protein lists (ratio of un-reviewed to reviewed proteins: AIS, 7/362; MIA, 7/389; LPIA, 6/717; surrounding, 4/166; frozen normal, 22/2103). We identified four proteins that have not been listed in the proteomics column of NextProt and PeptideAtlas.

Conclusion

This method is useful for enrichment of the highest PE representative proteins in protein inference lists that meet the requirement of the C-HPP Data Interpretation Guidelines.

Keywords

Protein inference, C-HPP Data Interpretation Guidelines

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Zika virus signature in fetal brains with microcephaly

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Background: Questions still unanswered are the association between ZIKV and microcephaly, the mechanisms of brain infection, the physiological processes affected – the ZIKV signature in the brain – and the possible presence of other etiological agents. A comprehensive understanding of these questions was approached by label-free quantitative shotgun proteomics of Zika-positive fetal brains with microcephaly as well as one Edwards´ syndrome control.
Method: Brain proteins were extracted with 5% deoxycholate, 0.75 mM dithiothreitol, protease and phosphatase inhibitors using a TissueRuptor. The 80% cold acetone pellet solubilized in 8 M urea/ 2 M thiourea was reduced, alkylated, trypsin hydrolyzed, and analyzed in four technical replicates in a nanoLC Easy 1000 coupled to a Q-Exactive Plus. Raw spectra were processed in SuperQuant node. Data search was performed against a concatenated all viruses and human entries, UniProt, Jan/17 by the Sequest HT algorithm. For FDR of <1% and label-free quantification we used Proteome Discovery 2.1.
Results: 4559 proteins were unique identified in four brains. Compared to the control, proteins of all ZIKV brains showed down-regulation of fibrinogen chains A and B and von Willebrand factor, LCP1 plastin and tropomyosin, transferrin, annexin 2, decorin and beta subunit of prolyl 4-hydroxylase as well as several collagen types and chains. Proteins up-regulated were microtubule associated protein 1 and tubulin, collapsing response mediator protein 1 and 2 and 3 dihydropyrimidinases as well as voltage dependent anion channels 1, 2, and 3, cytochrome c, pyruvate kinase, enolase, mitochondrial ATP synthase. This study identified as PE1 eight missing proteins still without SRM validation.
Conclusion: ZIKV infection of human developing brains dysregulates biological processes down-regulating hemostasis, cytoskeleton, iron transport, osteoclast formation and bone resorption, collagen metabolism, and extracellular membrane and cytoskeleton formation as well as enhances microtubule formation, axonogenesis and energy production.
Keywords: ZIKV, fetal brain, infection, pathology, quantitative proteomics.

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Lacrimal proteome: possible source of biomarkers?

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Background: The human tear is an easily and non-invasively collectible body fluid. It could be an excellent target for proteomic analysis for diagnosis and state monitoring of ocular and several systemic disease. The proteins of tear fluid has been secreted from the lacrimal gland or filtered from blood. The lacrimal gland is having neuronal connections with many area of the brain so the amount of the lacrimal gland secreted proteins could be an indicator of some psychiatric/neurodegenerative diseases also. Quantitative data on the amount, daily and personal variation of these lacrimal gland secreted and blood originated proteins in human tear are unknown. The composition of the human tear depends on several factors: personal differences, diseases, left/right eye dominance, part of the day, humidity etc. To develop a reliable diagnostic test for lacrimal proteome, the influence of these factors has to be determined.
In this study the daily change of protein profile and the personal variability were examined.

Methods: Because of the low protein content and volume of tear, the digestion method had to be optimized for small protein amount. This method is usable for reproducible digestion of 1 □g protein. All the LC-MS analysis were performed on a nanoUPLC-Quadrupole-Orbitrap hybrid MS system in DIA (Data Independent Acquisition) mode. For the quantification an optimized LC gradient and MS method were developed and iRT peptide standard kit (Biognosys) were used.

Results: High interpersonal, daily, and left/right eyes variability with significant differences in whole protein content and protein profile were detected.

Conclusions: Because of the high variability in concentrations of both low and high abundance proteins, a carefully designed normalization method is required for reliable statistical evaluation..

Keywords: tear fluid, biomarker, LC-MS, Data Independent Acquisition mode, quantitative proteomics

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MHC peptide ligands from influenza virus differ in their kinetics of presentation

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Background

Current vaccinations against influenza virus mainly induce antibodies against the viral surface glycoproteins hemagglutinin and neuraminidase. Unfortunately, these proteins strongly vary between influenza strains and are subject to rapid antigenic drift rendering the immunisation inefficient and requiring annually repeated vaccination. An alternative vaccination might aim at the induction of cytotoxic T cells directed against conserved or multiple viral epitopes providing efficient long-term antiviral immunity. However, T cells targeting major histocompatibility complex (MHC) class I ligands presented only at the end of the influenza virus lytic cycle might potentially kill their target cells only after viral release thus limiting their effectiveness.

Methods

The B cell line JY was infected with influenza virus A (H1N1pdm09), and MHC peptide ligands were extracted 0, 4, 8, 12 and 16 h post infection (p.i.). Mock infected JY cells were analysed in parallel. Obtained peptide samples were subjected to LC-MS employing the hybrid fragmentation method EThcD. Label-free quantification was performed with Skyline.

Results

We identified twelve influenza peptides predicted to bind the appropriate HLA-A*02:01 and HLA-B*07:02 MHC proteins. Five of these putative ligands showed sufficient MS1 signal for quantification. One MHC ligand from influenza NS1 protein could already be identified 4 h p.i., while three out of the five peptides were first detected 8 h p.i. A fifth peptide became detectable only 16 h p.i. Notably, the fifth peptide derived from the same source protein (hemagglutinin) as one of the three peptides appearing 8 h p.i.

Conclusions

The time p.i. at which half-maximal presentation of influenza MHC peptide ligands is achieved differs from peptide to peptide. This might have important consequences for the effectiveness of T cell responses in preventing influenza virus spreading in the body. Vaccinations aiming at cytotoxic T cells should therefore consider the kinetics of presentation.

Keywords

MHC, immunopeptidome, influenza virus, vaccination

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PHOSPHOPROTEOME AND SIALOME OF CELLS MODELLING TUMOUR INFILTRATING MYELOID POPULATIONS

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Background
Tumour infiltrating myeloid populations include macrophages and myeloid derived suppressor cells (MDSC) that expand in cancer bearing patients and infiltrate the tumour microenvironment promoting tumour progression and metastasis. Conventional anti-cancer treatments are aimed to arrest proliferation and cause death of tumour cells. However, the interaction between cancer cells and tumour-infiltrating myeloid populations hamper these therapies and suppress anti-tumour immune responses. A better knowledge of these subsets may lead to the development of more efficacious treatments by uncovering novel targets. The aim of this study was to characterise different subsets of MDSC and macrophages generated ex vivo which model tumour infiltrating counterparts and identify novel key targets that differentiate them from non-cancerous resting macrophages (M0).
Method
Tumour-associated macrophages (TAM) and MDSCs were differentiated ex vivo from murine bone marrow (BM) precursors using protocols established by our group. Macrophages (M0) differentiated from BM using medium supplemented with recombinant MCSF were used as controls. Proteins were extracted and digested, and peptides were subsequently labelled with TMT 10-plex to assess the differences between the three different myeloid cells populations. Phosphorylated peptides and N-linked sialylated glycopeptides were enriched by the TiSH protocol and fractionated by reverse phase at high pH and HILIC. All the samples were subsequently analysed by nLC-MS/MS using a Q-exactive MS.
Results
We identified multiple differences at protein expression levels between populations modelling tumour infiltrating subsets and M0. The differences between macrophages were more subtle than those between MDSCs and M0, and were fine-tuned by phosphorylation and sialylation. We found several significantly deregulated proteins involved in cell adhesion and migration, metabolic pathways, pre-mRNA processing/splicing, and protein translation. Activation of MAPK and CDKs were strongly associated to myeloid cells modelling tumour-infiltrating subsets.
Conclusions
We identified key pathways characteristic for tumour infiltrating populations that distinguishes them from resting macrophages.
Keywords
Myeloid cells, macrophages.

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Gender-enhanced alteration in airway epithelial proteome in COPD related to xenobiotic metabolism

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Background: Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of mortality worldwide. Smoking represents a major risk factor for COPD and evidence of significant gender differences with women having a higher risk to develop the disease than men are emerging. In this study, we investigated alterations in the airway epithelial proteome due to early stage COPD, with focus on gender-related differences.

Methods: The airway epithelial proteome from age- and sex- matched smoking COPD patients (COPD) (GOLD I-II/A-B), smokers with normal lung function (Smokers), and never-smoker healthy controls (Healthy) from the Karolinska COSMIC cohort were analyzed by Difference Gel Electrophoresis (n=85) and tandem mass tag-based shotgun proteomics (n=90). Multivariate statistical modelling and pathway enrichment analyses were performed stratified by gender- and proteomic approach.

Results: Significant gender-differences in the alterations of the epithelial proteome between Smokers and COPD groups was evident by both proteomics platforms, with distinct subsets of proteins providing significant classification of Smokers vs. COPD both in females ($p= 1.5\times10^{-4}$) and males ($p= 2.2\times10^{-5}$). Alterations were linked to dysregulation in protein folding in the ER, xenobiotic metabolism and oxidative phosphorylation. Proteins from the oxidative phosphorylation pathways correlated to CD8+ T-cells abundance in male COPD ($r=0.71$, $p<0.01$), and to lung density measured by computed tomography ($r=0.82$, $p<0.01$) and goblet cell density ($r=0.94$, $p<0.01$) in female COPD. Proteins involved in glutathione metabolism correlated with goblet cell density in female COPD ($r=0.77$, $p<0.01$).

Conclusions: This study shows gender-enhanced alterations in xenobiotic metabolism pathways in the airway epithelium in COPD. Associated gender-differences in the protection against noxious chemicals in tobacco smoke may be a link to divergent molecular COPD sub-phenotypes and differences in disease incidence between genders.

Keywords: COPD, proteomics, epithelial cells, gender differences, multivariate statistics

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Biomarkers to differentiate between CT-positive and CT-negative patients with mild traumatic brain injury

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Background. Brain lesions induced by a mild traumatic brain injury (mTBI) can be detected using CT-scan. However, CT-scans are expensive, harmful for the patients and 90% of all CT-scans are negative. Biomarkers as a decision tool to avoid CT-scans and quickly discharged patients have been widely studied. Despite several years of research on blood biomarkers, no effective alternative to the CT scan is yet commercially available. The most studied biomarker remains S100b with specificity around 25% and sensitivity close to 100%. Here, we performed protein discovery on blood and brain injury models to identify new potential biomarkers and investigated whether they could perform better than S100b and thereby avoid unnecessary CT-scans.

Methods. Biomarker discovery was performed by a mass spectrometry-based strategy using isobaric labelling (TMT-10plex) on plasma mTBI patients and a brain injury model (post-mortem CSF). Potential biomarkers were verified and validated by ELISA on a multicentric prospective cohort encompassing over hundred mTBI patients recruited within the first 6h after trauma. The patients were dichotomized into CT-negative and CT-positive groups for statistical analyses. The predictive performance of these biomarkers was established using Mann-Whitney U tests and ROC curves. PanelomiX was used for biomarker combination.

Results. The preliminary results identified a total of eight different proteins as potential biomarkers for mTBI; H-FABP, DJ-1, IL-10, MMP-1, CCL23, VCAM-1, SAA and GSTP. Three of these proteins, H-FABP, DJ-1 and IL-10, seem as efficient or even better than S100b to differentiate between CT-positive and CT-negative patients. A panel of H-FABP, S100b and age resulted with specificity above 50% for 100% sensitivity.

Conclusions. The present study identified 8 new biomarkers for mTBI diagnostics. The combination of H-FABP with S100b and age allowed to discharge more than half of the patients without brain lesion avoiding unnecessary CT-scans and save hospitalization costs.

Keywords. S100B, H-FABP, biomarker, mTBI

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Integration of Targeted Proteomics into Systems-Toxicology Approach: Candidate Modified-Risk Tobacco Product Assessment using Nasal Cultures

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Background

Systems toxicology complements standard toxicological endpoints with system-level measurements and computational analysis approaches. Here we show how targeted proteomics was used effectively in a system toxicology assessment study for the Tobacco Heating System (THS) 2.2, a candidate modified risk tobacco product (cMRTP), with human organotypic nasal cultures [1,2].

Methods

Human organotypic nasal cultures were exposed to 3R4F cigarette smoke (CS) or THS2.2 aerosol at similar nicotine concentrations, or fresh air (controls). Transcriptomic profiles were analyzed using gene set and causal network enrichment approaches. A protein-marker panel of relevant biological processes, such as xenobiotic metabolism, oxidative stress, and senescence, was quantified by parallel reaction monitoring (PRM). Pro-inflammatory mediators released into the medium were measured using antibody-based multi-analyte profiling.

Results

Analysis of the PRM marker panel supported that 3R4F CS elicited a xenobiotic metabolism, oxidative stress, and tissue adaptation response compared with fresh air. 3R4F CS also increased the release of pro-inflammatory mediators (IL8, IL6, TIMP1, MMP1). Consistent with the transcriptomics results, reduced alterations in the protein/gene expression and pro-inflammatory markers were observed in cultures exposed to THS2.2 aerosol compared with 3R4F CS at comparable nicotine concentrations.

Conclusions

Mass spectrometry-based (PRM) and antibody-based (multi-analyte profiling) targeted proteomics effectively supplemented the other endpoints within an in vitro Systems Toxicology assessment study for a candidate MRTP.

[1] A. R. Iskandar et al. 3-D nasal cultures: Systems toxicological assessment of a candidate modified-risk tobacco product, ALTEX, 2016, DOI: 10.14573/altex.1605041

[2] A.R. Iskandar et al. Systems Toxicology Meta-Analysis of In Vitro Assessment Studies: Biological Impact of a Modified-Risk Tobacco Product Aerosol Compared with Cigarette Smoke on Human Organotypic Cultures of the Aerodigestive Tract. Toxicology Research. In revision.

Keywords

Organotypic culture, parallel reaction monitoring, system toxicology

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Novel Chemoproteomic Characterization of Covalent Probe Binding

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BACKGROUND:
Despite the success of recent irreversible drugs, the possibility that covalent modification of unintended targets may lead to idiosyncratic toxicities remains a major concern. New approaches for proteome-wide identification of probe targets, including the site of covalent modification will facilitate development of covalent drugs.
METHODS:
We recently characterized unique gas-phase fragmentation for peptides modified by cysteine-directed covalent probes. We extended this framework to covalent probes elaborated with affinity handles and combined this new knowledge with multiplexed isotope reagents to provide a quantitative proteome-wide readout of concentration-dependent/-independent probe binding, based on identifying the specific, covalently modified cysteine residues.
RESULTS:
We incorporated desthiobiotin (DTB) onto a covalent inhibitor (THZ1) that targets CDK7, a kinase recently implicated as an oncogene. We incubated cells with different concentrations of native inhibitor (THZ1), followed by co-incubation with a fixed concentration of THZ1-DTB, pulldown, and further processing to yield tryptic peptides encoded with multiplexed isotope reagents. Quantitative mass spectrometry data comprised several hundred protein targets, each identified based on THZ-1-DTB modified cysteine residues. Analysis of the iTRAQ ratios distinguished concentration-dependent and –independent (e.g., highly-reactive) cysteine targets. We identified the intended target, CDK7, as well as suspected off-targets CDK12 and CDK13 as being bound in a concentration-dependent manner. We identified several unexpected off-target kinases that were selectively bound by THZ-1-DTB. We focused on PKN3, an understudied kinase which may play a functional role in metastatic prostate cancer.
CONCLUSION
We leveraged new insight for fragmentation behavior of covalent inhibitor-modified peptides with competition-format biochemical enrichment to provide proteome-wide identification of concentration-dependent probe binding. We readily identified unexpected targets for a previously credentialed, covalent inhibitor of CDK7. Our ability to directly identify the specific site of modification jump-starts medicinal chemistry campaigns for new probes, in our case against an obscure kinase PKN3 which may represent an attractive drug target.

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An in vitro MS assay for sensitive determination of donor specific MHC class II immunopeptidomes

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Background
The primary immune response to antigens is initiated through antigen presenting cells such as dendritic cells (DC). Major histocompatibility complex (MHC) class II associated human leukocyte antigens (HLA) on the surface of these cells present peptide fragments derived from proteins to CD4+ T helper cells which can initiate an adaptive immune response. The CD4+ T cells can drive activation of B cells presenting the same peptide fragment. Upon activation, B cells rearrange their immunoglobulin genes to produce high affinity, isotype switched antibody which binds to conformational epitopes on the same protein recognised by the T cells. This can lead to neutralisation of the therapeutic effects of protein-based therapies such as monoclonal antibodies.

Methods
Small numbers of human dendritic cells obtained from a cohort of donors were cultured in vitro in the presence of therapeutic monoclonal antibodies. The MHC II associated immunopeptidome was analysed using an affinity MS approach, employing a short one-dimensional reversed phase separation using a DIONEX Ultimate 3000 nano LC connected to a benchtop Orbitrap mass spectrometer (Thermo, QE plus).

Results
We have determined donor characteristic self-peptidome signatures and revealed peptides deriving from distinct sequence regions from constant and variable domains of the monoclonal antibodies present in the in vitro assay.

Conclusions
Donor individual immunopeptidome signatures as well characteristic peptide clusters derived from therapeutic monoclonal antibodies were accurately determined starting from a low number of in vitro cultured dendritic cells.

Keywords
Monoclonal antibody, Immuno-peptidomics, human dendritic cells, major histocompatibility complex (MHC), human leukocyte antigens (HLA).

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Global secretome analysis of human macrophages upon activation of innate immune response

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Background
The innate immune system is the first line of defense against microbial infections. It also responds to host factors that arise during tissue damage and metabolic dysregulation. Inflammasomes are multimeric cytosolic protein complexes that mediate innate immune responses to microbial infection, cellular damage, and metabolic dysregulation.
Protein secretion through multiple pathways is an important part of immune responses. Immune cells secrete for example small soluble proteins, cytokines and chemokines that are needed for proper coordination of host cell response to different pathogens. Recent system-level characterizations using proteomics methods have shown that immune cells activate a much more global protein secretion than just secretion of cytokines and chemokines.

Methods
We use human monocyte-derived macrophages and have studied global protein secretion upon different activation stimuli. These include viral, fungal, and bacterial infection as well as endogenous danger signals ATP and monosodium urate. We have analyzed total secretomes as well as isolated extracellular vesicles and used several proteomics workflows (iTRAQ, GeLC-MS/MS for identification and label-free quantification) combined with bioinformatics and functional studies.

Results
We have extensively used proteomics to globally characterize protein secretion from human macrophages upon different activation stimuli. Our studies have shown that activators of both canonical NLRP3 inflammasome as well as non-canonical caspase-4/5 inflammasome induce robust unconventional vesicle-mediated protein secretion in human macrophages. The secreted protein include many danger signal proteins, translation regulators, and proteins that are essential for cell signaling and adhesion.

Conclusions
Our studies have shown that unconventional vesicle-mediated protein secretion is an important part of the innate immune response against different microbial pathogens and molecules inducing tissue damage.

Keywords
Innate immunity
Protein secretion
Macrophage
Mass spectrometry

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Genetic wiring maps of single protein states reveal on off-switch for GPCR signaling

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Background
Using random mutagenesis in haploid human cells we apply a sensitive approach to directly couple genomic mutations to protein measurements in individual cells and complement this with proteomics to identify novel regulators of the AKT pathway.

Methods
Gene-trap mutagenized HAP1 cells were FACS-sorted after labeling with pAKT (S473) antibodies. Two cell populations, corresponding to the cells with the highest and lowest pAKT protein abundance, were isolated to determine gene-trap integrations by deep sequencing. Genes enriched for mutations in the 'high' and 'low' channel were subsequently determined as negative and positive pAKT regulators, respectively. Proteome and ubiproteome profiling were performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Ubiquitinated peptides were enriched using the PTMScan Ubiquitin Remnant Motif (K-ε-GG) Kit (Cell Signaling Technology). Raw data files were processed with MaxQuant using the LFQ algorithm.

Results
In addition to many known AKT pathway components, the cullin E3 ligase adaptor KCTD5 was identified as a potent negative regulator in the genetic screen measuring pAKT. Subsequent screens in ΔKCTD5 HAP1 cells suggested that AKT activation by G protein β and γ subunits (GNB1, GNB2 and GNG5) is suppressed by KCTD5/CUL3. LFQ proteome profiling revealed 70 proteins with increased abundance in ΔKCTD5 cells as compared to WT, whereas profiling of the ubiquitinated peptidome yielded 217 ubiquitination sites down-regulated in ΔKCTD5 cells. The overlap of up-regulated proteins and down-regulated ubiquitination sites comprised only 4 proteins, including GNB1, GNB2 and GNG5. Subsequent GNB1 screens in WT and ΔKCTD5 cells revealed that the Gα subunits GNAI1, GNAI2, GNAI3 and RIC8A specifically regulated GNB1 levels in wild-type cells.

Conclusions
Our data support a model where KCTD5 acts as an off-switch for GPCR signaling by triggering proteolysis of ubiquitinated Gβγ heterodimers dissociated from the Gα subunit.

Keywords
Gene-trap mutagenesis, FACS sorting, ubiquitinated peptides, pAKT, GPCR.

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Understanding the Molecular Effects of Antipsychotics on Oligodendrocytes through Phosphoproteomics and their relation with Schizophrenia

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Background: Schizophrenia is a chronic mental illness that affects over 1% of world's population. The diagnosis is still essentially clinical and the only way to control the symptoms is through the use of antipsychotics. The efficacy of these drugs has remained only low to moderate despite intensive research efforts. In addition, antipsychotic treatment can produce often-undesired, severe side effects in the patients and addressing these remains a large, unmet clinical need. One of the reasons for the low effectiveness of these drugs is the limited knowledge about the molecular mechanisms of schizophrenia, which impairs the development of new and more effective treatments. However, separate lines of investigations have demonstrated the involvement of NMDA receptor and oligodendrocyte dysfunctions in schizophrenia. Proteomic and phosphoproteomic studies have been used to understand the molecular basis of schizophrenia, as well as the mechanisms of antipsychotics in this disorder. Here we have carried out shotgun mass spectrometry proteome analyses of oligodendrocytes treated with the NMDA receptor antagonist MK-801 and antipsychotic medications to gain potential insights into these effects at the molecular level. Objective: This study aims to characterize the phosphoproteome of a human oligodendroglia cell line (MO3.13 cells) treated with pharmacological agents associated with schizophrenia and antipsychotics. Methods: MO3.13 cells were cultured and treated acutely (8h) with antipsychotics and with MK-801, an NMDA receptor antagonist. Sample preparation and phosphopeptide enrichment were performed using TiO_2. Finally, proteomic analyses were performed in a two-dimensional microUPLC coupled to nano ESI-Q-IM-TOF mass spectrometer. The data will be processed using PLGS 3.0.2 and Progenesis® QI for Proteomics 3.0 softwares (Waters). Expected Results: With generated data and functional correlation bioinformatic analyses, we expect to unravel possible disturbances in biochemical pathways in drug-treated cells. Data will help us to better understand the neurological mechanisms of antipsychotics in the pathophysiological processes of schizophrenia.

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Protocol optimization for deep analysis of Major Histocompatibility Complex (MHC) Immunopeptidomes

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Background
Over the last two decades, the discovery of T cell epitopes has been a promising approach to inform vaccine design for pathogenic infection, cancer, metabolic malfunctioning and autoimmune disorders. Different proteomics approaches have been used to identify and quantify the MHC-bound ligandome from cells, tissue and serum. Here we describe a systematic comparison of several MHC class I-associated peptide purification strategies and LC-MS/MS acquisition methods.

Methods
MHC-associated peptides were purified from Jurkat cells and MHC-associated peptides were analysed by LC-MS/MS using a Thermo Scientific Ultimate 3000 UPLC and Orbitrap Fusion Lumos instrument. Acquisition methods, different MHC-solubilisation approaches and complementary workflows to separate MHC-bound peptides from MHC protein complex components after immunoprecipitation were evaluated and compared for optimal performance.

Results
We conclude with a universal acquisition method for the analysis of immunopeptidomes on a Fusion Lumos instrument. Several solubilisation methods were identified as suitable for MHC-bound peptide extraction. The use of preparative HPLC for separation of peptide from larger complex components proved superior to molecular weight-based filtering techniques. Particularly pre-fractionation of the complex immunopeptidome sample can boost the number of identified MHC-ligands.

Conclusions
We conclude with a reliable and reproducible workflow for the identification of peptides bound to MHC class I molecules.

Keywords
MHC class I, Immunopeptidomics

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Systems biology of MHC class I antigen presentation studied in human cancer cell lines

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Background
Cancer is a primary cause of mortality in industrialized countries. Individualized medicine is regarded as a potential solution to low efficacies and high costs for innovation in drug development and health systems, but requires comprehensive data from individual samples and a well understanding of the underlying cancer biology. In the present study, we analyzed the proteome, transcriptome and ligandome of ten MHC class I matched human cancer cell lines derived from six different tissues with the aim to establish a prediction tool for MHC-presented peptide ligands, required for an antigen-specific immunotherapy.

Materials and methods
Tryptic peptides from tumor cell lysates were analysed by LC-MS using data-independent acquisition on a Waters Synapt G2-S HDMS instrument. LC-MS data analysis was performed in PLGS3.02 and ISOQuant. RNA analyses were performed with an Illumina HiSeq 2500 system. Reads were mapped against the human genome (hg19) using the STAR mapping Algorithm. MHC class I molecule associated peptides (ligandome) were isolated by immunoaffinity purification using W6/32 coupled BrCN-Sepharose beads.

Results
Using data-independent acquisition (DIA) label-free LC-MS, we established a reference proteome data set covering in total over 6.700 protein groups and their relative abundances. On mRNA level we identified between 9.791 and 12.467 expressed
(>= 1 RPKM) coding genes. Analyzing correlations between proteomics and transcriptomics data, we found high correlation values, ranging from r= 0.5 to 0.58 (spearman correlation coefficient). Gene Ontology analysis (protein folding, gene expression, RNA binding, etc.), revealed correlation values up to r= 0.89. Ligandome analyses identified between 762 and 3.338 ligands per cell line, the number of identified ligands correlated with the respective MHC expression level.

Conclusion
We generated comprehensive proteomic, transcriptomic and ligandomic data sets, which are integrated into a model to quantitatively predict the MHC class I ligandome and to establish novel strategies for antigen-specific immunotherapy.

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Identification of a damaging variant associated with idiopathic male infertility through degradation of TEX101 interactome

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Background: TEX101 is a testis-specific cell surface protein expressed exclusively in the male germ cells and is a validated biomarker of male infertility (BMC Medicine 2017, 15, 60). Molecular function of human TEX101 is not known, but it was suggested to act as a cell surface chaperone which regulates through protein-protein interactions maturation of numerous proteins involved in sperm-egg fusion and fertilization.

Methods: Since in vitro cell line models of human germ cells are not available, we studied TEX101 and its interactome in testicular tissues, spermatozoa and seminal plasma of healthy fertile men and patients with idiopathic infertility. Methods included DNA genotyping, ELISA, GluC-mediated SRM assays, immunofluorescence and co-immunoprecipitation-MS.

Results: TEX101 protein was measured in seminal plasma of 805 men, and rs35033974 variant was genotyped in spermatozoa DNA of 437 men. Measurements of TEX101 protein in the spermatozoa of wild-type, hetero- and homozygous men by GluC-mediated SRM assay revealed a near-complete degradation (>97%) of the G99V variant protein. Spermatozoa of patients homozygous for rs35033974 were thus used as a knockdown model to identify TEX101 functional interactome. Global proteomic profiling of spermatozoa from four wild-type (confirmed healthy fertile) versus four homozygous (confirmed idiopathic infertile) men quantified more than 8,000 proteins and revealed significantly reduced levels of numerous cell surface proteins involved in sperm migration and sperm-oocyte fusion.

Conclusions: Our study revealed that a common homozygous variant rs35033974 of TEX101 (~0.7% frequency in the general population) may be associated with idiopathic male infertility, and that the damaging effect of this variant is exerted through degradation of TEX101 protein and its interactome. Collectively, our findings may facilitate diagnostics of idiopathic male infertility and provide a rational selection of the most effective assisted reproduction treatments.

Keywords: Male infertility, damaging SNP, interactome, proteogenomics

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Reproducible Quantitative Proteomics of Extracellular Vesicles Released From Stimulated Brain Tumour Cells

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Background: Activated oncogenic pathways in cancer cells affect the release and cargo of extracellular vesicles (EVs) secreted by cancer cells. EVs can contribute to the transfer of oncogenes and their associated functional effects to recipient cells (Nat Cell Biol. 2008 May;10(5)). In this study, we use quantitative proteomics to probe the lysates of brain tumour cells and their EVs with and without growth factor stimulation.

Methods: To collect extracellular vesicles, the media was centrifuged (400g, 10 mins) and filtered to remove cellular debris. The media was then concentrated using a 100K centrifugal filter and ultracentrifuged at 110,000 x g for 70 minutes. uHPLC-MS/MS Parameters: Cell lysates or EVs were loaded onto a stacking gel. After reduction and alkylation, peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer. Database search results were loaded onto Scaffold Q+ for spectral counting statistical treatment. For XIC based MS1 quantification, *.raw data files search engine result files were loaded into Pinnacle for MS1 quantification.

Results
Over 2000 proteins were identified and quantified from cell lysates and released EVs. Three biological repeats were analysed and the statistically significant differences ($p=<0.05$) were overlaid on canonical biological pathways using the Reactome. Upon stimulation, the protein type and amount of the vesicular cargo changed substantially. Quantitative pathway analyses from brain cancer cells show that the basal levels of multiple cell signalling pathways and cell cycle proteins in the EV's are significantly altered after growth factor stimulation and appear to have more focussed signalling components and severely reduced amounts of cell cycle proteins.

Conclusions
Quantitative proteomics can detect and reliably quantify vesicular proteins. In addition, results overlaid on canonical pathway diagrams can be used to easily detect and examine changes to oncogenic cell signalling pathways.

Keywords : brain tumour cells MS1 quantification, extracellular vesicles, growth factor, oncogenic pathways

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Role of biomarkers in predicting healing of chronic venous ulcers: a proteomic approach

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Background: Chronic venous ulcers (CVUs) are associated with low quality of life, pain, exudate, odour, decreased mobility and self-esteem and contribute to patient withdrawal from professional activities. Difficult healing wounds adds extraordinary personal burdens, and increases financial costs to health systems. So, we searched biomarker proteins available in the inflammatory exsudate of CVUs, which they can predict the wound healing before the treatment. Methods: The inflammatory exsudate of 37 wounds of 28 patients treated or not with a new heterologous fibrin sealant were analysed by shotgun proteomic approach. The proteins were hydrolysed using trypsin enzyme and the triptic fragments were submitted to ESI-Q-TOF mass spectrometry. Statistical tests and Mascot, Scaffold and String softwares have assisted to identify, characterize and validate the biomarkers. Results: Seventy-six proteins were revealed, which act as transportation, immunomodulation, matrix protein, proteases inhibitors, genetic components and microbial activity. The correlation between clinic variables and proteome profile showed the extensive association of Apoliprotein-A1 with the ulcer areas. Sixty-seven percente of wounds not treated presented reduction of area in relation to the evolution time. Shotgun analysis indicated ten proteins diferentially express such as Complement-C3 and Ceruloplasmin related to fast cicatrization of the wounds, while Apoliprotein-A1 and Neutrofil defensin-1 related with bad cicatrization. When wounds were treated with a new heterologous sealant fibrin, 72.1% cicatrized or reducted the area. Complement C3/C4-B, Neutrofil defensin-1 and alpha-2-macroglobulin presented diferential expression in the inflammatory exsudate after this new treatment. Conclusions: We suggest that Apoliproteína-A1, Complement-C3 and Neutrofil defensin-1 are potential candidates to prognostic/diagnostic biomarkers of CVUs cicatrization. So, this work can assist to predict the evolution of wound healing when a patient with CVUs looks for treatment in the Dermatology. Keywords: Chronic venous ulcers, biomarkers, mass spectrometry, shotgun, heterologous fibrin sealant.

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Comparison of protocols for the processing of proteomics samples that are limited in quantity

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Background
Several protocols have been introduced for the digestion and processing of proteomics samples, including Filter Aided Sample Preparation (FASP), Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) and in-Stage Tip (iST) digestion. We carried out an independent comparison between the three different methods and assessed their performance for the processing of proteomic samples in the low µg range.

Methods
HeLa cell lysate (20-1 µg of protein) was digested using either SP3, FASP or iST (commercial kit from PreOmics GmbH (Martinsried, Germany)). Tryptic digests were analyzed by LC-MS analysis on a Synapt G2-S HDMS mass spectrometer (Waters) coupled to a nanoAcquity UPLC system (Waters). Data were acquired in DIA (UDMSE) mode. Data processing and analysis was performed in PLGS 3.02 (Waters) and ISOQuant.

Results
We focused on the preparation of starting material in the low µg range (20 µg -1 µg of total protein). While all methods showed comparative performance using 20 µg of starting material, performance of FASP dropped significantly below 20 µg of starting material. Processing 5 - 20 µg of protein, iST outperformed both, FASP and SP3 in terms of identified peptides and proteins. In the lower µg range (1 - 2 µg), however, SP3 and iST showed very similar performance. When digesting 1 µg of starting material, both methods still enabled the identification of around 3,000 proteins and between 25,000 to 30,000 peptides. On average, the quantitative reproducibility between technical replicates was slightly higher in case of SP3 (R2= 0.97 (SP3); R2= 0.93 (iST)). SP3 performance was also assessed for the handling of FACS sorted macrophages and enabled the quantification of 3,000 proteins from 30,000 cells.

Conclusions
The SP3 protocol introduced by Hughes et al. (2014) outperformed FASP and iST when limited amounts of starting material (1 µg of proteins) were available.

Keywords
Sample preparation

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Quantitative analysis of protein, mRNA and miRNA response to hypoxia in primary hippocampal neurons

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Background

Hypoxia poses a challenge for any organism that is dependent on oxygen as electron acceptor. The local availability of oxygen inside an organism can change due to reasons ranging from pathologies such as solid tumors, ischemia and inflammation to growth during embryonic development. A fast response is necessary to prevent damage to cells and tissues. Here, we investigated primary hippocampal neurons after hypoxia. Quantitative proteomics and sequencing of mRNA and microRNA allowed simultaneous investigation of proteins, miRNAs and mRNAs, as well as the interplay between them.

Methods

Primary cultures were grown from murine hippocampal neurons and maintained in normoxic conditions until treatment. Hypoxic conditions (1% O_2) were applied and cells harvested. Proteins were isolated and tryptically digested. Measurements for label-free quantitative mass spectrometry were carried out on an Eksigent nanoLC425 hyphenated to a Sciex TripleTOF 5600+ operated in data-independent mode for SWATH acquisition. Data was analyzed using ProteinPilot and PeakView software packages. Sample preparation for RNA-Seq was performed using the TruSeq RNA Sample Prep Kit. Sequencing data from the Illumina HiSeq2000 platform was analyzed using in-house bioinformatics pipelines.

Results

Hypoxia led to stabilization of the hypoxia-inducible factor 1-alpha (HIF1-α) and upregulation of miR-210 in primary hippocampal neurons. These responses to hypoxia are known from other tissues on the proteomic and miRNA level, respectively. Furthermore, SWATH analysis allowed for quantification of 2016 proteins. Clusters of up- and downregulated elements were identified for proteins, mRNA and miRNA.

Conclusions

The comprehensive data sets gathered for protein, mRNA and miRNA from primary hippocampal neurons enable insights into the molecular response to hypoxia for this specific cell type. Besides the direct impact on the proteome and mRNA levels, regulatory effects of miRNAs can be elucidated by merged analysis of all three levels.

Keywords

SWATH, RNA-Seq, Hypoxia, miRNA, Hippocampal neurons

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Serine Palmitoyltransferase Protein Interaction Landscape and Structural Characterisation

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Background:
Serine palmitoyltransferase (SPT) catalyses the first step in de novo sphingolipid biosynthesis. SPT condenses L-serine and palmitoyl-CoA to produce 3-ketodihydrosphingosine (KDS), a precursor to sphingosine, ceramides, sphingomyelin, and glycosphingolipids. The human enzyme is a complex of three proteins (LCB1, LCB2, and ssSPT) and is endoplasmic reticulum (ER) membrane bound. Mutations in SPT cause human sensory neuropathy type 1 (HSAN1) resulting in progressive neuronal degradation. SPT mutants have been shown to synthesise toxic deoxy-sphingolipids from alanine and glycine rather than serine, although the exact pathological mechanism is not known. Regulation of SPT is not well understood and the protein interaction landscape has not been fully characterised.

Methods:
BirA(R118G)-SPT gene fusions were used for biotin proximity labelling to isolate interacting proteins from both human and yeast cells. The human SPT complex was also expressed and purified from the yeast ER as a single gene fusion. Purified protein has enabled kinetic studies as well as structural analysis using lysine reactive crosslinking reagents.

Results:
Known and novel putative SPT interactors have been identified through biotin proximity labelling. A gene ontology analysis of interactors highlighted membrane organisation and ER-golgi transport. Activity assays confirm that purification of the fusion-enzyme in n-dodecyl-beta-maltoside micelles yields active protein. Lysine-lysine crosslinking and mass spectrometry analysis gives support to a structure modelled on the bacterial SPT homodimer.

Conclusions:
Putative protein interactors suggest SPT may play an important role in membrane organisation and transport, or alternatively, SPT regulation may be mediated by enzyme localisation. Independent analyses are underway to validate putative SPT interactors. Additionally, in the absence of a crystal structure, crosslinking has provided a crucial insight into the structure of the SPT complex.

Keywords:
serine palmitoyltransferase, protein interaction, biotin proximity labelling, crosslinking

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A Core Facility Approach to Affinity Purification-Data Independent Analysis for Interactome Analysis

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Background
Protein-protein interaction analysis has greatly benefited from mass spectrometric development. Affinity purification-mass spectrometry (AP-MS) enables the identification or verification of unknown protein interactors or neighbours on a routine basis. Recently, Data-Independent Analysis (DIA) was introduced as part of AP-MS workflows. Compared to existing methods based on e.g. Spectral Counting, DIA offers increased reproducibility at high analytical depth, and should thus increase statistical significance of AP-MS results. We tested different AP-DIA workflows and software packages, with a special view to implementation in an academic core facility. Results obtained on affinity-purified Nup85 NPC subcomplexes from Xenopus laevis were evaluated for analytical depth, reproducibility, time/cost efficicency and results dissemination.

Methods
Affinity-purified complexes were reduced, alkylated and trypsinized after SDS-PAGE fractionation, after SDS-PAGE cleanup without fractionation or digestion in-solution. Samples were analysed by RPC18-nLC/MS/MS using both Data-Dependent Acquisition and Data-Independent Acquisition on a hybrid QqToF mass spectrometer (TripleTOF 5600+, Sciex). Following protein identification, SWATH quantitation was achieved using PeakView 2.2 (Sciex), Spectronaut 8.0 (Biognosys), Skyline (UWash) and OpenSWATH (ETH Zürich).

Results
Testing different sample preparation, data acquisition and processing strategies against an established Spectral Counting workflow we observed the following: (i) preparation can be performed by either SDS-PAGE cleanup and in-gel digestion or by in-solution digestion. Fractionation provided no extra benefit; (ii) best performing spectral libraries were obtained by DDA acquisition of AP sample and control. DDA analysis of AP input provided no benefit; (iii) of the available software, PeakView and Spectronaut both provided a balance of quantitation performance with ease of use and reporting.

Conclusions
AP-DIA is a highly efficient approach to protein-protein interaction analysis. By optimization of sample preparation, library building strategy and software we established a routine workflow for AP-DIA that is time and cost efficient in the framework of a core facility, and produces statistically reliable interaction data.

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Combatting drug resistance in Multiple Myeloma

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Background
Multiple myeloma (MM) is a debilitating malignancy that is part of a spectrum of diseases ranging from monoclonal gammopathy of unknown significance (MGUS) to plasma cell leukaemia. It is estimated that about 86,000 incident cases occur worldwide annually, accounting for about 0.8% of all new cancer cases, with 5-year relative survival rates of 50%. Over the past two decades, novel therapeutics options including proteasome inhibitors (PI) and immunomodulatory drugs (IMiDs) are changing the treatment paradigm in MM and improving overall survival. Despite these advances, acquired or intrinsic resistance to therapy leads to disease progression, and novel treatment strategies are urgently needed.
Drug resistance is the major problem for the effective treatment of MM as not all patients' respond and even in responding patients, complete remissions are infrequent or relapse occurs. There are a number of mechanisms of drug resistance including reduced intracellular drug accumulation, altered drug distribution within the cell, enhanced ability to repair drug damage and inhibition of apoptosis. Overcoming drug resistance is very complicated as cancer cells can adapt with multiple mechanism of resistance active at any time. Therefore, it is necessary to address multiple mechanisms in order to overcome drug resistance completely.
Results
We have identified a number of key intracellular pathways found to be significantly altered using ex vivo CD138+ purified patient plasma cells screened against standard of care oncology drugs.
Conclusion
Mechanisms underlying drug resistance in MM are not completely understood and as new therapeutic options become available, it will be essential to identify the patients that harbour intrinsic resistance mechanisms and identify acquired mechanism in patients receiving specific treatment. Proteomics is a promising strategy to analyse the protein expression profiles in drug-resistant MM cells and provide potential new therapeutic targets and clinically useful biomarkers.
Keywords
Multiple myeloma, CD138+, drug resistance, proteomics

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An Organoruthenium Anticancer Agent Shows Unexpected Target Selectivity For Plectin

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Organometallic metal(arene) anticancer agents require ligand exchange for their anticancer activity and this is generally believed to confer low selectivity for potential cellular targets. However, using an integrated proteomics-based target-response profiling approach as a potent hypothesis-generating procedure, we found an unexpected target selectivity of a ruthenium(arene) pyridinecarbothioamide (plecstatin) for plectin, a scaffold protein and cytolinker, which was validated in a plectin knock-out model in vitro. Plectin targeting shows potential as a strategy to inhibit tumour invasiveness as shown in cultured tumour spheroids while oral administration of plecstatin-1 to mice reduces tumour growth more efficiently in the invasive B16 melanoma than in the CT26 colon cancer xenograft.

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Differential Proteomic Analysis of Metformin Response in a Preoperative Window Clinical Trial for Endometrial Cancer

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Background: The preoperative use of metformin in obese women with endometrioid endometrial cancer (EC) is associated with reduced tumor proliferation and an inhibition of the mammalian target of rapamycin (mTOR) pathway in select cases. We sought to evaluate whether a proteomic signature exists to explain the inhibition of mTOR and to identify a biomarker that may predict response to preoperative metformin, using samples from our previously completed phase 0 study of metformin in obese EC patients.

Methods: Differential proteomic analysis of laser microdissected tumor cells collected from matched, pre-treatment biopsies and post-treatment hysterectomy EC patient tissue specimens (n=20) treated with metformin (850 mg) daily for 1-4 weeks prior to surgical staging was conducted. Thirteen patients responded and seven patients did not respond to metformin treatment based on decreased expression of the proliferation marker Ki-67. Mass spectrometry-based proteomics was performed on tryptic digests of tissue samples. Protein abundance was measured using spectral counting, and functional analyses were performed using Ingenuity Pathway Analysis.

Results: The proteomics analyses identified 79 significantly (edgeR $p<0.05$) altered proteins between metformin responder and non-responder patients that suggested increased activation of signaling pathways promoting cell proliferation and inhibition of pathways associated with cellular apoptosis. We further identified hematological and neurological expressed 1 (HN1) as being markedly abundant in metformin responder versus nonresponder patients. HN1 was further found to be significantly decreased in metformin responder patients following differential analyses of post- vs. pre-treatment tissue proteomic data.

Conclusions: Differential proteomic analyses revealed alterations of signaling pathways supporting increased cell proliferation and decreased cellular apoptosis signaling in metformin responder vs. non-responder EC patients. Protein HN1 was significantly elevated in responder vs. non-responder patients and further decreased in comparisons of post- vs. pre-treated responder patients. HN1 represents a predictive biomarker candidate of preoperative therapeutic response to metformin in EC patients.

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THE CITRULLINOME IN TISSUE AND BIOFLUIDS FOR DISEASE PHENOTYPING AND PERSONALIZED MEDICINE

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Background
Protein citrullination is fundamental to several essential processes in apoptosis and antimicrobial defense, however, also linked to multiple pathogenic endpoints. This post-translational modification (PTM), by conversion of arginine to citrulline residues, is mediated by peptidylarginine deiminase (PAD) enzymes found in specific cells and tissues. Autoantibodies against citrullinated proteins (ACPAs) is a diagnostic feature for multiple autoimmune diseases.

Methods
Synovial fluid (SF) and plasma was collected from patients diagnosed with RA, OA, SpA (n=120). Intestinal tissue (colon mucosa) from RA patients (n=10) and joint lysate from collagen-induced arthritis mouse model (n=24). All samples were analyzed by citrulline specific sample preparation and high-end mass spectrometric analysis. Follow-up studies were performed by multiple techniques including confocal microscopy, inflammation profiling, cell-free DNA measurement and optimized ACPA Immunome protein arrays.

Results
The proteome analysis of tissue and biofluids allowed deepest proteome analysis of SF so far (Proteoforms >1300) as well as a large number of citrullinated sites. The three patient groups could be differentiated by cluster analysis and the occupancy of each modified site calculated. The investigation of the intestinal tissue enabled identification of 223 citrullinated peptides from 121 proteins. Three of the peptides were unique to RA. The list of citrullinated proteins was enriched in extracellular and membrane proteins and included known targets of anticitrullinated protein antibodies (ACPAs). We developed an ACPA Immunome protein array (1536 target proteins) enabling subtyping of RA patients based on plasma reactivity for PAD isoforms.

Conclusions
Our deep proteome based analysis of tissue and biofluids have enabled an extended catalogue of citrullinated proteins and sites relevant to improved disease subtyping. The development of ACPA Immunome protein array will enable a potential diagnostic platform for personalized and precision medicine.

Keywords
Citrullination, Protein array, post-translational modification, autoimmune disease

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Rapid, Rugged, and Reliable Screening Methods for Serum Peptide Degradomics Profiling

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Background
Serum peptidome profiling has shown tremendous potential for identifying novel biomarkers. However, standard collection and preparation methods result in post-collection variance, reducing the sensitivity for reproducibly profiling donor samples based on differential profiling of putative biomarker panels.
Methods
The proposed method dilutes whole blood in guanidine prior to resting and low molecular weight separation and analysis. A set of whole blood samples were divided into different lots, with and without guanidine dilution and then underwent similar sample preparation, data acquisition, and profiling. LC-MS analysis was performed using a Q Exactive mass spectrometer with HRAM MS quantitation and supporting HCD fragmentation for sequencing. A detailed spectral library was acquired on a pooled sample and long gradients and used to process all routine samples eluted off a SPE column and 5-minute injection cycle. Additional analyses were performed on stratified donor samples between normal and diseased for additional method evaluation.
Results
Sample preparation using guanidine dilution and off-line peptidome extraction showed the greatest peptidome coverage with the lowest variance. Over 70% of all target peptides had coefficient of variation less than 15% compared to the set of samples without guanidine dilution prior to preparation. In addition, the high resolution and accurate mass analysis on the Q Exactive facilitated reproducible profiling for over 1000 peptide targets in the five minutes, utilizing gas-phase fractionation. Differential profiling of the donor samples identified a set of 85 putative markers that are being further evaluated for biological relevancy.
Conclusions
Incorporation of guanidine for immediate sample dilution and washing, helped reduce post-collection degradation that can increase the biological noise and reduce reproducibility. The creation of the detailed spectral library also increased efficiency of tandem MS data profiling, increasing the number of targets sampled in 5 minutes.

Key Words
Degradomics, global profling, high resolution/accurate mass, high-throughput

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SwathXtend modules to improve protein detection confidence using SWATH-MS with large peptide spectral reference libraries

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There is increasing interest in the use of data-independent acquisition (DIA) strategies in proteomics to avoid undersampling and provide more reliable run-to-run peptide quantification to support large-scale studies. One implementation of DIA is known as SWATH-MS where there are two main steps: generation of a reference peptide MS/MS assay library by conventional data-dependent acquisition followed by SWATH data acquisition and peak area extraction of individual samples. The breadth and depth of MS/MS spectra in the reference library crucially influences the final SWATH quantitative result and it is becoming increasingly common to utilise pre-existing reference libraries rather than build individual libraries on a per case basis. Inherent in this approach are bioinformatics workflows to ensure high quality data integrity.
Our previous work introduced SwathXtend, a bioinformatics package for merging SWATH libraries to enable searching of large repositories without requiring iRT peptides to LC calibration. In this study we present a new module which introduces a new range of filters and thresholds to scrutinize the extended libraries, increase the protein detection confidence and ultimately improve the reliability of SWATH comparative proteomic analyses. There are three major components in this workflow: library filtering, generation and checking, SWATH data extraction, and results filtering and comparing. For assay libraries generated from databases with many redundant entries, our workflow incorporated a deconvolution approach which exploits the extraction of shared and non-shared peptides combined with a post-extraction peptide to protein mapper to assign protein areas into unique protein and shared protein groups. We demonstrate the workflow on a published SWATH plasma dataset using an extended library with a publicly available human plasma assay library and a SWATH dataset generated from Thyroid cell lines using an assay library containing protein isoforms . This workflow is applicable to any SWATH experiments regardless of sample type.

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Profiling Of Proteoforms In Human Tears Using Chip-Based Capillary Electrophoresis Coupled To Mass Spectrometry

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Introduction
Tear fluid is of key importance to maintain the health of the front of the eye and provide clear vision. In addition, it represents a promising body fluid that can help in the diagnosis and prognosis of various eye (as well as metabolic and even neurological) diseases. Here we use top-down proteomics combined with CE to map the major proteoforms in the tear fluid.

Method
Tear samples were taken using Schirmer's strips. The strip was placed into the lower eye lid for 3 min or until 70% of the strip surface was wetted by tear fluid. Protein were extracted by submerging each strip into a 10% acetonitrile, 1% formic acid solution in water and shaking for 5 min. Extracted proteins were directly transferred to an injection vial and analyzed by CE-MS using a modified Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

Results
Our workflow includes the combination of Schirmer strips for sample collection, sample handling, and protein extraction in conjunction with fast CE-MS profiling in less than 1 hour. Samples were analyzed over a 10 min CE separation by MS and MS/MS. One of the proteins represented by a multitude of proteoforms is lacritin, which is a prosecretory mitogen capable of promoting basal tearing and which low levels are associated with dry eye syndrome. Overall, this strategy offers a powerful option for discovery and characterization of potential tear biomarkers that could be used for the screening of diseases, both eye related or other.

Conclusion
Fast characterization of tear proteoforms in minutes on an Orbitrap-based mass spectrometer.

Keywords
Top Down, ZipChip, CE, clinical proteomics, translational research

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Novel stool-based protein biomarkers for improved colorectal cancer screening: a case-control study

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Background
The fecal immunochemical test (FIT) is used in many countries for non-invasive screening for colorectal cancer (CRC), but its characteristics leave room for improvement. We aimed to identify novel stool-based protein markers in stool that outperform or complement hemoglobin in detecting CRC.

Methods
A total of 313 stool specimens (91 CRC, 40 advanced adenoma (AA), 43 nonadvanced adenoma (A) and 139 controls) were analyzed by in-dept mass spectrometry GeLC-MS/MS workflow (Q-Exactive). CART analysis and logistic regression revealed combinations of 4 proteins as the most optimal combination to differentiate CRC from controls. Antibody assays (MSD) were performed on a validation set of 72 FIT fluid samples set (14 CRC, 16 AA, 18 A and 24 controls).

Results
Of the 468 human proteins quantified in the discovery set, 93 were significantly enriched in CRC vs controls ($p<0.05$). Of these, 29 were significantly found enriched in CRC vs controls in a validation set ($q<0.05$). Receiver operation characteristics analysis of a combination of four proteins detected CRC with a sensitivity of 73% as compared to 43% for hemoglobin (HBA1) alone at 95% specificity ($p=0.00003$). Similarly a combination of four proteins was identified for differentiation of AA from controls, which showed a sensitivity of 48% vs 8% for hemoglobin ($p=0.0002$) at 95% specificity. Selected proteins could be measured in small sample volumes used in FIT-based screening programs, and discriminated CRCs from controls ($P<0.001$).

Conclusions
Proteome profiling on stool samples revealed 29 validated proteins significantly enriched in CRC samples compared to controls. A panel of four complementary protein markers outperformed hemoglobin for detection of CRC as well as AA. Proof-of-concept for detecting the proteins in FIT fluids confirmed the high potential of these markers for screening purposes in a non-invasive and cost-effective manner.

Keywords
Colorectal cancer, Early detection, protein biomarker, Stool, LC-MS/MS

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Time-resolved proteome and phosphoproteome analysis to dissect pemetrexed induced signaling cascade in non-small-cell lung cancer

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Background

Pemetrexed (PEM), an antifolate agent, is used as the first-line treatment for advanced non-small-cell lung cancer (NSCLC) patients. Despite the known mechanism on three targeted enzymes, the more comprehensive understanding of drug induced signaling pathways is still unclear. Thus, we aim to investigate a time-course molecular mechanisms induced by PEM in NSCLC.

Methods

A549 lung adenocarcinoma cells were treated with PEM for 30 min, 1h, 2h and 6h. Total proteins were purified, subjected to in-solution digestion and labeled with different TMT tags. The TMT-tagged peptides were separated into two parts; one applied high-pH reversed phase StageTip fractionation for proteome analysis, another applied IMAC purification for phosphoproteome analysis. Both were analyzed in duplicate by LC-MS/MS. Proteome Discovery was used for protein identification and quantitation with 1% false discovery rates. Functional analysis was applied by using DAVID and IPA software.

Results

The proteome analysis quantified 6,572 proteins in 4 time-course treatments, and the phosphoproteome analysis quantified 5,120 phosphopeptides corresponding to 1897 phosphoproteins in all treatments. The functional/pathway analysis of integrated results indicated G1 phase arrest and apoptosis in 30-min treatment, and activation of both caspase-dependent and –independent apoptosis induced by TNFR/FAS in 30-min, 2-h and 6-h simultaneously. The DNA damage induced ATM up-regulation further triggered DNA repair by up-regulation of BRCA1/BRAD complex 1 in 2-h and 6-h treatments. Also, CD27-CD70 signaling was upregulated in 2-h and 6-h treatments which might further induce regulatory T cells (Treg) proliferation to promote angiogenesis and tumor growth.

Conclusion

We provided a systematic molecular investigation in PEM-induced cancer cell killing mechanisms. Based on these results, we proposed that sequential combination regimens of PEM with anti-CD70 mAb therapy or AZD0156 targeting ATM that might provide a synergistic therapeutic effect in NSCLC.

Keywords:

Quantitative proteomics, phosphoproteomics, pemetrexed, NSCLC

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Early-Stage Blood-Based Proteomic Diagnosis of Colorectal Cancer

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Background: Colorectal cancer (CRC) is the 2nd most common cause of cancer-related mortality worldwide. 5-year survival rates of CRC patients found with early tumors is >90% following surgical resection. Low abundance proteins released directly from the tumor or tumor microenvironment into plasma may provide a source of minimally-invasive diagnostic/prognostic biomarkers and aid in our understanding CRC pathobiology.

Methods: A comprehensive exploratory study was performed using SWATH MS on 100 patient plasma samples (i.e., pools n=20 for each of 4 CRC stages: Dukes' stage A-D) against healthy, age- and sex-matched individuals (also pooled from n=20 healthy individuals). For deeper insights into the plasma proteome, pooled plasma samples were doubly ultradepleted using an in-house IgY chicken antibody depletion method (depletes ~ 200 high to medium abundance plasma proteins) and commercially-available Agilent MARS-14 system that depletes the 14 most abundant plasma proteins.

Results: Statistical analysis was performed on technical triplicates run on SWATH. Out of 250 quantifiable proteins, 6 protein candidates F2, HGFAC, PON1, CST3, ADAMDEC1 and CFD exhibited differential expression across all CRC stages compared to healthy controls. We are currently progressing towards target validation for all 6 candidates. We have successfully established a CST3 western blot assay that validates decreased expression in the earlier stages of CRC in pooled plasma as was observed by SWATH-MS.

Conclusions: The Human Protein Atlas illustrates that ADAMDEC1 mRNA is highly expressed in small intestine, rectum and colon. CST3 and PON1 has been reported as downregulated proteins in later stages of rectal cancer, thereby making the 6 plasma protein candidates we discovered as MRM assay targets for CRC early diagnosis prior to accurate confirmatory colonoscopy followed by surgery. Future work will involve quantification and validation of identified targets on individual plasma samples and development of immuno-MRM based assays.

Keywords: Colorectal cancer, plasma, SWATH-MS, DIA, immuno-MRM

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Exposure to metals during pregnancy and adverse birth outcomes: Mechanistic insights from the MIREC study

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Environmental exposure to elevated levels of some metals during pregnancy is associated with low birth weight and preterm birth. Nevertheless, chemical exposure-related toxicity mechanisms that precipitate these adverse birth effects are not clearly understood. Also, heavy metals are known to display endocrine disrupting properties. The aim of this work was to identify metal exposure-related changes in maternal pathways that may lead to adverse birth outcomes, through the use of proteomic and metabolomic biomarker analyses. Third trimester maternal plasma samples from a Canadian mother-infant (Maternal-Infant Research on Environmental Chemicals -MIREC) study were analysed for target proteomic/metabolomic markers (multiplex protein array, HPLC-Fluorescence, HPLC-Coularray and EIA). Statistical tests were conducted to test associations between maternal blood metal (Cd, Hg, Pb, As, Mn) levels, plasma biomarkers, physiological changes and birth outcomes. Associations ($p<0.05$) were seen between maternal blood As, Hg, Pb concentrations and third trimester maternal plasma matrix metalloproteinases (MMPs), key enzymes in the process of pregnancy. Maternal blood metal levels also exhibited dose-dependent changes in oxidative/nitrative stress pathways. MMPs, vascular endothelial growth factor, cellular adhesion molecules, and chemokines relevant to inflammatory/vascular pathways were associated ($p<0.05$) with small or large for gestational age. Our results suggested phenotypic heterogeneity based on targeted maternal plasma proteomic and metabolomic markers among the smaller and larger birth weight for gestational age groups that resembled potential infection, activated inflammation or vascular mechanisms. These findings suggest that future high-content proteomic, metabolomic and epigenomic/genomic studies will be useful in advancing our understanding of the environmental metal exposure-mediated molecular mechanisms that may adversely affect infant birth weight and down-stream health effects later in life.

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Discovery and Targeted MS-based strategies for the identification of glaucoma-related IgG V domain peptides.

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Background:
Glaucoma is a neurodegenerative ocular disease and one of the primary causes of blindness worldwide. Autoantibody profiling has attracted a lot of attention in the research field of glaucoma promising new biomarker candidates for diagnostic purposes in future. Main objective of this study was to identify and validate glaucoma-related peptides from the variable IgG domain employing discovery and targeted MS strategies.
Methods:
Individual sera samples were taken from 13 patients diagnosed with primary open-angle glaucoma (POAG, N=13, mean age=60±7) and 15 healthy controls (CTRL, N=15, mean age=56±5). IgG were isolated by anti-Fc (crystallisable fragment) resin beads and digested into Fab (antigen-binding fragment) and Fc by papain. Fab was further purified from the digested mixture by anti-Fc resin beads followed by In-solution trypsin digestion. Signature peptides revealed by untargeted discovery proteomics ($p \leq 0.05$) were targeted by LC-MS in each individual run via accurate inclusion mass screening (AIMS) strategy. Label-free quantitative statistics was performed in order to validate glaucoma-specific marker candidates.
Results:
Statistical analysis of the discovery proteomics data revealed 124 peptides of the variable IgG domain to be significant differently expressed between POAG and CTRL group using two-sided t-test ($p \leq 0.05$). Up to 7 peptides were high abundant in the POAG group, whereas the majority of 117 peptides was low abundant. AIMS strategy verified 35 targeted IgG V domain peptide sequences showing decreased expression levels in the POAG group in comparison to CTRL ($p \leq 0.05$). Exactly 9 of these peptides were assigned as complementarity-determining regions (CDR) of the variable IgG domain.
Conclusion:
Combination of shotgun proteomics for discovery and targeted MS for verification were suitable for the identification of highly diverse IgG V domain peptides as potential biomarker candidates in glaucoma sera.

Keywords: IgG; CDR; Glaucoma; Autoimmunity

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All-inclusive proteoform profiling of serum proteins by hybrid mass spectrometry approaches

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Many proteins in human blood plasma are decorated by a plethora of post-translational modifications (PTMs). These modifications affect the functionality and clearance of the protein and therefore need to be characterized in detail. Plasma glycoproteins also represent some of the most relevant protein biomarkers. Next to changes in abundance, changes in PTM/glycosylation profiles may provide strong biomarker signatures. Here we present detailed structural analysis combining primarily two advanced mass spectrometry (MS) based methods; high-resolution native MS and peptide centric proteomics of several human blood serum-derived proteins involved in the immune response via complement activation. Following purification from plasma, highly resolved proteoform profiles of the intact glycoproteins were acquired by high-resolution native MS using a modified Exactive Plus Orbitrap EMR instrument. This data revealed qualitatively and quantitatively the co-occurrence of tens to hundreds of proteoforms of the studied plasma glycoprotein. Subsequent glycopeptide centric analysis by LC-MS using CID/HCD/ETD and EThcD provided site-specific quantitative profiles of all PTMs. An integrative data processing provided in unprecedented detail a superior characterization of the plasma glycoproteins. Our data reveal new insights in the structural heterogeneity of biologically important plasma glycoproteins, exposing also several novel PTMs. For example, we discovered unreported O-glycosylation sites on complement component C9 and exposed the unexpected presence of an additional N-glycosylation site out of the canonical N-glycosylation sequon. We also revealed unexpected heterogeneity in occupancies of C-mannosylation on factor P. We examined several other proteins involved in complement activation. Furthermore, we introduce an efficient algorithm that allows a direct comparison of the data obtained from the two independent methods (native MS and peptide centric MS). This algorithm can also be used for defining biosimilarity between closely related proteins, which would be beneficial for stratifying therapeutic biosimilar/biobetter proteins

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Functional Lipidomics: Palmitic Acid Impairs Hepatocellular Carcinoma Development by Modulating Membrane Fluidity and Glucose Metabolism

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Lipids are essential cellular components and energy sources of living organisms, altered lipid composition is increasingly recognized as a signature of cancer. We performed the lipidomic analysis in a series of hepatocellular carcinoma (HCC) cells and identified over 1700 intact lipids originating from 3 major lipid categories. Comparative lipidomic screening revealed 93 significantly changed lipids and decreased palmitic acyl (C16:0)-containing glycerophospholipids (GPs) were positively associated with metastatic abilities of HCC cells. Furthermore, both in vitro and in vivo experiments designated that C16:0 incubation specifically reduced malignant cell proliferation, impaired cell invasiveness, and suppressed tumor growth in mouse xenograft models. Biochemical experiments demonstrated that C16:0 treatment decreased cell membrane fluidity and limited glucose metabolism. Phosphoproteomics approach further revealed such C16:0 incubation attenuated phosphorylation levels of mTOR and Stat3 pathway proteins. MRM analysis showed 8 declined C16:0-containing lipids out of total 10 changed lipids when compared cancer tissues with adjacent nontumor tissues in a cohort of clinical HCC specimens ($P<0.05$). Conclusion: These data collectively herald the biomedical potential of using altered lipid metabolism as a diagnostic marker for cancerous cells and open an opportunity for treating aggressive HCCs by targeting the altered C16:0 metabolism.

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mass-spectrometrists should search for all peptides, but assess only the ones they care about

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Background
In shotgun-proteomics identified mass-spectra deemed irrelevant to the scientific hypothesis are often discarded, e.g. when focusing on proteins in specific pathways or few species in metaproteomic samples.
To improve statistical power, Noble (2015) urged researchers to remove irrelevant peptides from the database prior to searching rather than (1) matching all peptides to spectra (PSMs), (2) calculating the false discovery rate (FDR) and (3) filtering irrelevant reliable PSMs (conventional method).
We argue that both the conventional as well as the Noble-method lead to poor FDR control.

Methods
We propose the search-all-assess-subset (saas) method that (1) searches against all expected proteins, and (2) discards irrelevant PSMs prior to (3) FDR calculation.
Because all-sub combined with the target-decoy approach (TDA), suffers from unstable FDR estimates in small subsets with few decoys, we also suggest to estimate the distribution of incorrect PSMs using a large decoy set.

Results
We compare the conventional method, the Noble-method and all-sub in a simulation study, a human-protein-contaminated Plasmodium case-study, and a Pyrococcus case-study where subsets are defined using GO-terms.
We show that the conventional method leads to invalid FDR control because it removes PSMs post FDR calculation returning either too conservative or too liberal subset-PSM lists.
The Noble-method calculates the FDR on the subset but puts a burden on TDA by forcing many high quality spectra with better matches in the complete search on subset sequences: often the fraction of forced PSMs even out-numbers the FDR level.
Hence, the FDR control is questionable at best.

Conclussion
0ur strategy returns more PSMs than the Noble-method and provides correct FDR-control.
Hence, mass-spectrometrists benefit from searching for all proteins and only assessing the ones they care about by using our web tool (http://iomics.ugent.be/saas/) or R package (https://github.com/compomics/search-all-assess-subset).
(preprint: https://doi.org/10.1101/094581)

Keywords
FDR; complex samples; Target-Decoy-Approach

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Quantitative phospho–proteomics in the mouse liver uncovers diurnal regulatory landscapes

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Background
Reversible post-translational modifications (PTMs) of proteins such as phosphorylation and acetylation play a key role in many essential cell-signaling networks. Diurnal oscillations of gene expression controlled by the circadian clock enable organisms to coordinate physiology with daily environmental cycles. While crucial insights into regulation at the transcriptional level has been established, much less is known about temporally controlled functions at the protein level in the nucleus.
Methods
We performed a circadian proteomic and phospho-proteomic analysis of the mouse liver nucleus. We quantified the temporal nuclear accumulation of proteins and phosphorylated proteins using in vivo stable isotope labeling by amino acids in cell culture (SILAC). Samples were analyzed with liquid chromatography tandem mass spectrometry (LC MS/MS). MS/MS data was processed with MaxQuant and Andromeda as search engine against the UniProt database restricted to mouse (Mus musculus) taxonomy and a custom database containing usual contaminants. Protein identifications were filtered at 1% FDR against a reversed sequence database.

Results
Thousands of nuclear proteins were identified. In total, over 500 proteins showed a diurnal accumulation rhythm. Parallel analysis of the nuclear phospho-proteome allowed us to infer the temporal activity of kinases accounting for rhythmic phosphorylation. Many of the proteins identified were parts of nuclear complexes involved in rhythmic transcriptional regulation, among other important metabolic pathways.

Conclusions
Optimized sample preparation/enrichment and LC MS/MS combined with SILAC enabled large-scale quantitative proteomics. Our findings provided unprecedented insights into the diurnal regulatory landscape of the mouse liver nucleus. These results are relevant to future chrononutrition-based therapies but also provide new hypotheses far beyond the circadian field. We have demonstrated that high-throughput quantitative nuclear phospho-proteomics is crucial to discover new pathways and mechanisms in the nuclear compartment and can be generalized to different models and systems.
Keywords
SILAC, Post-translational modification, Mass spectrometry, Regulation, Nuclear proteins, Transcription, Cell cycle.

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Complete Native SILAC of Prototrophic Yeast for Proteome-wide Quantification

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SILAC is one of the most widely used stable isotope labeling techniques for accurate proteome-wide quantification by MS. Usually, a combination of stable isotope-coded 'heavy' arginine and lysine is employed for labeling. Using trypsin for protein digestion results in maximum quantitative information since virtually all proteolytic peptides are labeled. However, prototrophic organisms such as baker's yeast are able to synthesize all amino acids on their own. This compromises complete SILAC-labeling of yeast and, thus, accurate proteome quantification by MS. In addition, heavy arginine may be converted to heavy proline, affecting quantification accuracy. To circumvent these issues, appropriate auxotrophies are introduced into yeast. 'Native SILAC' is a strategy to label prototrophic yeast under defined conditions, but current protocols are restricted to lysine labeling only. We extended native SILAC to the use of both heavy arginine and lysine and showed its high applicability for accurate quantitative proteome studies in baker's yeast.

Yeast were labeled with heavy arginine/lysine or lysine only in the presence of different carbon sources and under defined growth conditions. Proteins were in-gel digested with LysC, trypsin or both and quantitatively analyzed by LC-MS.

Prototrophic yeast show high incorporation efficiencies (~98%) and low arginine-to-proline conversion (<5%) following native SILAC using heavy arginine/lysine. We established the strategy for yeast grown on different fermentable and non-fermentable carbon sources and show that arginine/lysine labeling is superior to labeling with lysine only regarding protein identification, relative quantification, and sequence coverage. We applied this complete native SILAC approach to global quantitative analyses of yeast strains deficient for distinct mitochondrial components.

Our generic, complete native SILAC strategy allows for directly using and systematically screening yeast mutant strain collections available to the scientific community, which are usually arginine and/or lysine prototrophic, to address a multitude of biological questions requiring different carbon sources.

native SILAC, prototrophic yeast, mitochondria

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Quantitative phosphoproteomic analysis reveals shared and specific targets of Arabidopsis MAPkinases MPK3, MPK4 and MPK6

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Background: In Arabidopsis, mitogen-activated protein kinases MPK3, MPK4 and MPK6 constitute essential relays for a variety of functions including cell division, development and innate immunity. While some substrates of MPK3, MPK4 and MPK6 have been identified, the picture is still far from complete.

Methods: To identify the substrates of these MAPKs in cell division, growth and development we compared the cytosolic and chromatin-linked phosphoproteomes of wild-type and mpk3, mpk4 and mpk6 mutant plants. To study the function of these MAPKs in innate immunity, we analyzed their phosphoproteomes following activation by a microbe-associated molecular pattern (MAMP).

Results: We identified 152 differentially phosphorylated peptides in cytosolic fractions, in response to MAMP treatment and/or when compared between genotypes. 70 of these could be classified as putative MAPK targets, with phosphosites that are specific to one or shared by several MAPKs. Biochemical analysis of a number of putative MAPK substrates by phosphorylation and interaction assays confirmed our global phosphoproteome approach. Finally, we examined in detail the unknown function protein AYL1 (AT5G43830), confirming that it is a MAPK target and plays a role in defense responses. The results obtained on the fraction of chromatin-linked phosphoproteins confirm the picture obtained in the cytosol: in particular, the most dramatic effect on the phosphoproteome is observed in the mpk6 deletion mutant.

Conclusions: Partially overlapping substrate networks were retrieved for all three MAPKs, showing target specificity to one, two or all three MAPKs in different biological processes. Interestingly, we unveil the fact that within a given protein substrate, different phosphosites may be modified by one specific MAPK or by several of the three MAPKs. Our study also expands the set of novel MAPK substrates and functions to other involved protein kinases, including calcium-dependent (CDPK) and sugar non-fermenting (SnRK) protein kinases.

Keywords: phosphoproteomics, MAPKinase, immunity, signaling, Arabidopsis

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COSS: Free, Fast and powerful CompOmics Spectral Search Tool

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Background

Spectral similarity searching to identify peptide-derived MS/MS spectra is a promising technique, and different spectrum similarity search tools have therefore been developed. Each of these tools, however, comes with some limitations, mainly due to low processing speed and issues with handling large databases. Furthermore, only specific spectral data formats are typically supported, which also creates a threshold to adoption. We have therefore developed, COSS, a new user-friendly spectrum library search tool that relies on the probabilistic scoring function of Andromeda.

Methods

First, a study of existing tools was performed to understand the issues that came up. Next, a new spectrum similarity search tool, COSS, was developed in Java that tries to avoid the issues encountered with existing tools. This new tool was then tested on known data sets and its performance compared with another well-known existing tool.

Results

We have developed a user-friendly spectrum library search tools that is capable of handling large data sets and that is quite fast. We have compare the result with SpectraST on the same data set and achieved better performance.

Conclusions

There is a need for spectral library search tools that can study data from today's high-throughput mass spectrometry-based proteomics experiments, and that can match the tens of thousands of acquired spectra against proteome-wide spectral libraries. A few such search algorithms have already been developed, but come with limitations: the long search time or inability to handle very large data sets. We here present a new spectral library search tool that is fast, that can handle large data sets, and that supports commonly used spectral data formats.

Keywords: Spectral library, identification, Scoring-function, Optimization.

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THE NIPPLE ASPIRATE FLUID IN HEALTH AND DISEASE

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Background: Nipple aspirate fluid (NAF) is produced by epithelial cells lining ducts and lobules, which are most commonly associated with 85% of breast carcinomas. We used a proteomics approach to determine if NAF has the potential to be a liquid biopsy for detecting diagnostic biomarkers of diseases such as cancer.
Methods: NAF samples were collected from breast cancer patients and healthy volunteers at Bradford Teaching Hospitals NHS Trust, between 2013 and 2016. Matched pairs of NAF samples from 4 individuals (a healthy volunteer and patients with benign tumour, ductal carcinoma in situ [DCIS] and invasive carcinoma [IC]) were trypsin digested, separated by offline strong cation exchange chromatography, followed by reverse phase HPLC on a Dionex Ultimate 3000 separation system coupled to Orbitrap Fusion mass spectrometer. Label-free quantification was performed using Proteome Discoverer and protein identification by Mascot, database searching of the SwissProt database.
Results: 2D LC/MS proteomics of the eight samples identified 1990 unique gene products, with 567 common to all samples. Pairwise comparison of the proteomics profiles indicated a strong correlation between matched pairs (Pearson correlation coefficient values averaging 0.94). The healthy patient NAF samples had a high composition of milk proteins, but had not breast fed, and was indicative of galactorrhea as a side effect of prescription drugs. Comparison of the healthy and benign profiles with the cancer cases (DCIS and IC) indicated a significant increase in stromal, cell adhesion and basement membrane proteins indicating an escalation in tissue disruption.
Conclusions: Differentiating the diseased breast from the contra-lateral healthy breast will be challenging due to the similarity in profiles. However, the changing levels of cancer associated proteins in individuals with different stages of the disease warrants further diagnostic evaluation.
Keywords: Breast Cancer; Liquid biopsy; Nipple Aspirate Fluid (NAF), Comparative Proteomics.

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Refining Human proteome: Integrated analysis of human tissues with a multi-omics approach

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Background:
Having established an initial human proteome draft, we are now generating an integrated multi-omics map of 30 different healthy tissues. The integrated analysis of comprehensive and deep proteome, transcriptome, and additional external resources reveals novel insights into tissue-specific biology covering, for instance, proteome complexity, isoform expression, translational regulatory mechanisms as well as drug discovery aspects.

Methods:
Fresh frozen tissues were lysed and tryptically digested in solution using a urea-based procedure. The desalted digest was fractionated into 36 hydrophilic-SAX fractions and analyzed on a Q Exactive Plus with HCD fragmentation. Identification and label-free quantification of peptides and proteins was performed using MaxQuant and Mascot with Ensembl and customized proteogenomic databases. mRNA sequencing was performed on Illumina HiSeq2000 and 2500 machines.

Results:
Thirty different human tissues have been profiled to a depth of 15,000 to 17,000 transcripts and 9,000 to 11,000 protein groups per tissue, amounting to a total of around 16,000 protein groups. Overall, the number of truly tissue-specific proteins is in the range of a few hundred proteins, while around 8,500 proteins are expressed at similar levels across many tissues.
In a proteogenomics effort of a selected tissue (tonsils) using a multi-protease/multi-fragmentation approach, we identified 9,898 and 4,522 high-quality missense mutations on exome and RNA-Seq level, respectively, while only 2,048 variant peptides by proteomics, which are currently being validated using synthetic reference peptides.
To further understanding of translational and post-translational control, we analyzed the protein/mRNA ratio for individual genes across many tissues. Interestingly, about 4500 genes show fairly consistent protein/mRNA ratios (within a 5x range). Within the experimental uncertainty, this indicates that their synthesis and degradation are similarly balanced under steady-state conditions in different tissues.

Conclusions:
The present study provides a consistent proteomic and transcriptomic baseline map of 30 tissues.

Keywords:
Multi-omics analysis, human proteome, transcriptome, proteogenomics

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Understanding differential signaling via toll like receptor‑2 by multipronged quantitative proteomics

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Background: Emerging evidence suggests that one of the major immune evasion strategies used by M. tuberculosis is its ability to evoke a deliberate Th2 response which in turn down-regulates the anti-mycobacterial Th1 response. Differential expressions of cytokines such as IL-12 p40 and IL-10 by macrophages are known to dictate the development of Th1 and Th2 T cell responses and thereby determine the course of infection to a great extent. Earlier, we reported that the PPE18, a member of the PPE family of proteins of M. tuberculosis upon interaction with TLR2, can induce IL-10 production and anti-inflammatory responses, whereas another mycobacterial protein, PPE17, upon interaction with the same receptor, induces TNF-α production and proinflammatory signaling cascades. The disparate activities of the PPE17 and the PPE18 protein could be due to their ability to recognize different stretches of the TLR2 ectodomain, i.e. LRR motifs 11–15 by PPE18 and 15–20 by PPE17, resulting in differential modulation of post-receptor binding events that leads to activation of NF-κB transcription factors.

Methods: Label-free; iTRAQ labeling, SCX, and OFFGEL fractionation; Nano-LC-Orbitrap–MS/MS; Functional pathway and network analysis, Real-time PCR

Results: Using quantitative proteomics, we demonstrated that these two PPE proteins induce different signaling downstream of TLR2, that lead to a change in the protein kinase C (PKC)ε–IL-1R–associated kinase (IRAK3)– MAPK phosphatase 1 (MKP-1) signaling axis, which is involved in regulation of the TLR2-dependent inflammatory responses in macrophages.

Conclusions: Our study hints to a novel mechanism that implicates PKC«–IRAK3–MKP-1 signaling in the regulation of MAPK activity and inflammatory cascades downstream of TLR2 in tuberculosis. This may have an important impact in designing effective immunomodulators to trigger protective immune responses to control tuberculosis and various intracellular infections.

Keywords: Quantitative proteomics, TLR2, M. tuberculosis, PPE proteins

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Identification of Functional Protein Biomarkers from Cancer Patient Serum with PEP Technology

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Proteins play essential roles in numerous biological processes and being able to pinpoint functional differences between cell or tissue samples can greatly aid in understanding disease processes and metabolic changes. The PEP technology allows systematic analysis of protein functions within a proteome. Hundreds of functional proteins can be separated and functionally assayed to generate a comprehensive three-dimensional landscape of protein families such as protein kinases, phosphatases, proteinases and oxido-reductases. This information can then be integrated into other genomic and proteomic knowledge bases to provide further insight of important biological processes such as cancer development and aging.
In the PEP technology, complex protein mixtures are first separated by a modified two-dimensional gel electrophoresis process, giving enhanced resolution while still maintaining protein function. This is followed by an efficient protein transfer to a specially designed 1536-well Protein Elution Plate. After further transfer of the samples from the PEP plate to multiple 384-well microplates, functional assays can be performed on each well to generate an enzyme activity profile displayed in 3-D. Protein components of each well can be further characterized by mass spectrometry if desired.
Using the PEP technology, functional biomarker candidates have been identified from lung and breast cancer patient serum. Both qualitative and quantitative differences of metabolic enzymes and proteases were observed when comparing the cancer patient serum and normal serum. Some of the active enzymes were identified by mass spectrometry and validated in selected bioassays. It is believed that this functional proteomics technology provides a unique approach in the discovery of potential cancer biomarkers for diagnostic and prognostic applications.

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Increased Efficiency for the Preparation of Complex Proteomic Samples

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Efficient protein digests, produced in as little as 30 minutes, were prepared using a novel Rapid Digestion kit containing either a modified Trypsin (active/stable at elevated temperatures) or Rapid Trypsin/Lys-C mix. The protocol does not involve any immobilized enzymes, does not require denaturants or surfactants, and can be scaled as needed. Proteolysis can occur over a wide temperature range although the target temperature is typically 70 °C. Our initial time-course studies focused on optimizing variables including digestion time, temperature, concentration of reducing and alkylating agent, and enzyme substrate ratio. A temperature of 70 °C, was found to be an optimal proteolysis temperature with more mixtures reaching an optimal number of protein/peptides within 30-90 minutes. Other temperatures can also be implemented since the buffer was optimized to increase digestion efficiency, over a wide temperature range (25-70 °C). Reduction/alkylation was not required for all sample types and this step was found to work effectively when performed concurrently with the proteolysis step. We have further found that the protocol is compatible with denaturing agents like urea, although this requires lower temperatures due to the well-known decomposition of urea. The use of denaturants is particularly important when preparing membrane fractions. Other sample preparation modifications, including acetone precipitation, will also be discussed. The consistency of sample preparation was examined using both Label-Free Quantification as well as Tandem Mass Tags (TMT reagents).

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A personalized model of cholesterol homeostasis mechanisms based on trans-omics data integration

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Background
In individuals, heterogeneous drug response phenotypes result from a complex interplay of dose, specificity, genetic, and environmental factors. This challenges our understanding of the underlying mechanisms and optimal use of drugs in the clinic. Here, we present an approach that combines mass spectrometry (MS)-based proteomics and metabolomics with mechanistic modeling to characterize and explain context-dependent cellular drug response in different cell lines challenged with the same drugs. The approach was applied to drugs affecting cellular cholesterol regulation, a biological process with high clinical relevance.
Methods
Four model cell lines were challenged with targeted pharmacologic (drugs: statins and LXR agonists) or genetic (siRNAs) treatments and the elicited response phenotypes were quantified by SWATH-MS (ABSciex 5600) or on a flow-injection platform (Agilent 6550 Q-TOF) respectively. Using a logic-based modeling approach, the quantitative data from in total 491 MS-injections was used to generate cell line-specific core regulation networks of cholesterol homeostasis.
Results
The quantitative data from up to 6000 different proteins, metabolites, or phosphopeptides showed that each cell line has a specific basal biomolecular abundance profile that is perturbed in characteristic ways. The cholesterol synthesis pathway was affected in the expected direction and other metabolic pathways not previously known to be regulated by these drugs were identified. The four cell line-specific core regulation networks can explain in detail the processes beneath the observed intracellular drug response, identify the differences between the cell lines, and predict the effect on cholesterol uptake, a clinically relevant process that was not directly measured.
Conclusion
We present here an efficient approach that combines proteomic and metabolomic measurements with mechanistic modeling to understand the effect of drugs on complex biological processes. The generation of such cell line-specific regulation models represents an important step towards an accurate prediction of context-dependent personalized drug response.

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AAgAtlas 1.0: a human autoantigen database

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Background
Autoantibodies refer to antibodies that target self-antigens, which can play pivotal roles in maintaining homeostasis, distinguishing normal from tumor tissue, and trigger autoimmune diseases. In the last three decades, tremendous efforts have been devoted to elucidate the generation, evolution and functions of autoantibodies, as well as their target autoantigens. However, reports of these countless previously identified autoantigens are randomly dispersed in the literature.

Methods
Here we constructed an AAgAtlas database 1.0 using text-mining and manual curation. We extracted 45,830 autoantigen-related abstracts and 94,313 sentences from PubMed using the keywords of either ‘autoantigen’ or ‘autoantibody’ or their lexical variants, which were further refined to 25,520 abstracts, 43,253 sentences and 3,984 candidates by our bio-entity recognizer based on the Protein Ontology.

Results
We identified 1,126 genes as human autoantigens and 1,071 related human diseases, with which we constructed a human autoantigen database (AAgAtlas database 1.0). The database provides a user-friendly interface to conveniently browse, retrieve and download human autoantigens as well as their associated diseases. The database is freely accessible at http://biokb.ncpsb.org/aagatlas/.

Conclusions
We believe this database will be a valuable resource to track and understand human autoantigens as well as to investigate their functions in basic and translational research.

Keywords
Autoantigen, antoantibody, database, proteomics, disease

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Multiplexed Nucleic Acid Programmable Protein Arrays

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Background
Cell-free protein microarrays display naturally folded proteins based on just-in-time in situ synthesis and avoid the manipulations inherent in traditional expression, purification, printing and storage approaches. They have found increased acceptance in the past decade and led to important contributions in basic and translational research. However, during in situ expression, some intermediates are untethered to the surface, creating the potential for diffusion that has limited the final feature density on the array.

Methods
Here we utilized a different strategy to produce high density arrays that do not require any specialized equipment or substrates. We developed the Multiplexed Nucleic Acid Programmable Protein Array (M-NAPPA) method by multiplexing DNA plasmids (i.e., > 1 different plasmids within one spot). Using M-NAPPA, the number of displayed proteins can be increased up to five-fold, which significantly decreases the time, cost, labor, reagents, and sample consumption for high-throughput target discovery and verification.

Results
Even when proteins of widely varying sizes were displayed at the same feature, they were readily detected on M-NAPPA arrays using protein specific antibodies. Protein-protein interactions and serological antibody assays using human viral proteome microarrays demonstrated that comparable hits were detected by M-NAPPA and non-multiplexed NAPPA arrays. The last, combining M-NAPPA with a photolithography-based silicon nano-well platform enabled the creation of an ultra-high-density tuberculosis proteome microarray, which displayed entire tuberculosis proteomes on a single microscope slide and demonstrated the applicability of M-NAPPA philosophy on different microarray platforms.

Conclusions
These combined results demonstrated that multiplexing features on a protein microarray offers a cost-effective fabrication approach and has potential in facilitating high throughput translational research.
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Keywords
Protein array, Nucleic Acid Programmable Protein Array (NAPPA), protein-protein interaction, serological antibody
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Comparative proteomic study of CD34 hematopoietic stem cells from HbE/beta thalassemia

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β-Thalassemia/hemoglobin E (HbE/β-thal) is a compound heterozygous autosomal recessive hemolytic disease commonly found in Thailand, which is due to reduced production of the normal β-globin chain of hemoglobin, together with Hb E, resulting in an imbalance between β- and α-globin chains. The unmatched α-globin chains cause damage to membranes of erythroid precursor cells within the bone marrow resulting in ineffective erythropoiesis as well as circulating red blood cells, resulting in their reduced lifespan. However, the mechanism of ineffective erythropoiesis in Hb E/β-thal has not been well addressed. In order to gain further insight into this process, a proteomic approach was used to investigate the differential protein expression profile during in vitro development of CD34 multipotent hematopoietic stem cells obtained from bone marrow aspirates of 3 Hb E/β-thalassemia patients and 3 normal subjects. Extracted proteins were separated by one-dimensional SDS-PAGE, visualized by silver staining, in gel tryptic digestion then subsequently subjected to MS/MS analysis. The label free quantification method using Decyder analysis was performed the comparative differential protein expression of HSCs from patients and donors at day 0 and day 7. Interestingly, over expression of proteins involving the oxidative stress including PRDX2, PRDX6, CAT, and SOD1 were identified only in HbE/β-thalassemia HSCs. We postulate that increased oxidative stress in developing Hb E/β-thal erythroid precursor cells (under culture conditions) compared with normal controls results in both accelerated cell proliferation as well as apoptosis. These results suggest that enhanced apoptosis over proliferation may contribute to the ineffective erythropoiesis of Hb E/β-thal disease.

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MS/MS Strategies to Characterize The Proteoform Landscape In Human Tears Using Top-Down Mass Spectrometry.

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Introduction

Tear fluid is of key importance to maintain the health of the front of the eye and provide clear vision. In addition, it represents a promising body fluid that can help in the diagnosis and prognosis of various eye (as well as metabolic and even neurological) diseases. Here we use top-down proteomics to map the proteoform diversity in the tear fluid.

Method

Tear samples were taken using Schirmer's strips. The strip was placed into the lower eye lid for 3 min or until 70% of the strip surface was wetted by tear fluid. Proteins were extracted by submerging each strip into a 10% acetonitrile, 1% formic acid solution in water and shaking for 5 min. Extracted proteins were directly transferred to an injection vial and analyzed by LC-MS using an Orbitrap Fusion Tribrid Instrument modified with an UVPD source.

Results

Our workflow includes the combination of Schirmer strips for sample collection, sample handling, and protein extraction in conjunction with an array of top-down fragmentation techniques on a chromatographic time scale that allows for identification of over 50 proteoforms simultaneously in less than 1 hour. Samples were analyzed over a 30 min LC gradient by MS and MS/MS using various fragmentation mechanisms. One of the proteins represented by a multitude of proteoforms is lacritin, which is a prosecretory mitogen capable of promoting basal tearing and which low levels are associated with dry eye syndrome. Overall, this strategy offers a powerful option for discovery and characterization of potential tear biomarkers that could be used for the screening of diseases, both eye related or other.

Conclusion

The use of multiple MSMS fragmentation strategies allows for the characterization of tear proteoforms in minutes on an Orbitrap Fusion Tribrid modified with an UVPD source.

Keywords

Top-Down, UVPD, clinical proteomics, translational research

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Impact of alternative splicing on the cellular proteome quantified by targeted proteomics

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Background

The central dogma describes the flow of genetic information from DNA to RNA and protein. However, it makes no statement about the quantitative relationships between transcript and protein concentrations due to the complex post-transcriptional mechanisms. Alternative splicing is one of these mechanisms and engenders cellular proteomic diversity through the formation of a multitude of transcript and protein product(s) from the same locus. This diversity also affects the organization of the proteome into functional modules or protein complexes. However, these notions have not been quantitatively tested.

Methods

We significantly perturbed RNA splicing by depleting the core spliceosome U5 snRNP component PRPF8 in Cal51 breast adenocarcinoma cells. We used RNA-sequencing to comprehensively report intron retention, differential transcript usage (DTU) and gene expression and SWATH mass spectrometry (SWATH-MS) to capture an unbiased and quantitative snapshot of the impact of constitutive and alternative splicing on the proteome diversity. We then integrated the two data dimensions. Precise selective reaction monitoring (SRM) with labeled peptides spiked was used for further confirmation.

Results

We identified 388 genes displaying DTU after PRPF8 depletion. SWATH-MS quantified 419 peptides corresponding to 75 DTU genes, yielding an mRNA-protein Spearman's correlation coefficient R of 0.49 (P=1.97E-05). With SRM, 35 peptides corresponding to 15 DTU genes were measured, yielding an R of 0.78. Using antibodies we experimentally verified a functional switch event of LAP2 after PRPF8 depletion, whereby the dominant isoform changes from LAP2β (localized at nuclear lamina) to LAP2α (at nuclear interior). Furthermore, those alternative splicing events causing retained introns (270 genes) were found to harbor decreased protein abundance (P=0.0041). The on-going work focuses on resolving protein complexes for studying the impact of alternative splicing in proteome organization.

Conclusions

Our analysis demonstrates that alternative splicing events significantly contribute to both proteomic composition and diversity.

Keywords

mRNA-protein correlation, Alternative splicing, SWATH-MS, SRM

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Proteogenomic approaches to identify alternatively splicing variants in pancreatic cancer cell lines

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Background
Identification of alternatively splicing variants (ASV) is one of the major goals of the C-HPP, because they can increasing the diversity of human proteome and affect many biological mechanisms. Although protein sequence database is required for MS-based peptide identification, many of them cannot fully support sample specific ASVs. Thus, we employed proteogenomic approaches to produce protein sequence database containing splicing variants from RNA-seq. Given that Complement factor B is known as one of the emerging diagnostic markers of pancreatic cancer, we applied this database to identify differentially expressed proteins and ASVs present in the CFB silenced PANC-1 cell lines (Panc1-shCFB). Here we report identification of those ASVs in Panc1-shCFB cell lines.

Methods
Extracted RNAs obtained from each Panc1-Mock and Panc1-shCFB cell lines were subjected to RNA-seq by Illumina HiSeq2500 sequencer. Assigning each RNA-seq read to reference genome by TopHat2 and make protein sequence database using QUILTS. MS/MS spectra of proteins in each cell line were obtained using Q-Exactive MS analysis and proteins were identified in accordance with HPP Data Interpretation Guidelines.

Results
Using the RNA-seq data from each cell line, we were able to construct cell line specific ASV DBs. This DB contains total 456,667 novel ASVs which have not been previously annotated in Ensembl or neXtProt. The newly identified ASVs will not only help us understand how CFB exerts its multiple biological functions in pancreatic cancer but also identify those proteins involved in pancreatic cancer diagnosis.

Conclusions
Using the proteogenomic approach, we constructed the sample specific novel ASV sequence databases from RNA-seq dataset. This can be applied to identification of CFB regulated proteome including ASVs. We anticipate that this approach can identify novel ASVs from the biological samples which can be applied to detect sample specific changes in splicing variants at the levels of RNA and ASV expression

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A site-specific O-GlcNAc of tumor suppressor FoxO3a serves as a molecular signature of pancreatic cancer

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Background
Pancreatic ductal adenocarcinoma (PDAC) is one of the worst malignant tumors which is usually asymptomatic and the case of operable cancer after diagnosis is less than 15%. Unfortunately, there is no early diagnostic marker at present. PDAC can be caused by many factors including a loss of function mutation of tumor suppressor genes (e.g., FOXO). Here we explored how changes in O-GlcNAcylation of FoxO3a function as molecular signature of cancer progression in PDAC.

Methods
For identification of O-GlcNAc modification within FoxO3a, human tissues, including PDAC (n=24), were used with IRB approval of Yonsei University. Pancreatic cancer cell lines were purchased from ATCC. Nano LC–MS/MS analysis was performed with nano-chip HPLC.

Results
Following detection of increased levels of O-GlcNAc at FoxO3a in PDAC tumors, we further identified seven O-GlcNAcylation sites of FoxO3a in PANC1 cells using mass spectrometry (MS). Of these sites, serine 284 (S284) appeared to be critical to the tumor suppressor activity of FoxO3a, as S284A mutant cells (in PANC-1 cell) lacking O-GlcNAcylation exhibited an increased expression of p21, resulting in arrest of PANC-1 cell growth. Furthermore, treatment of PDAC cells with cancer drugs not only reduced the levels of the O-GlcNAc of FoxO3 in HPAC cells but also activated p21 expression. Proteomic quantification of S284A mutant cells revealed markedly different protein profiles compared to those in PDAC cells.

Conclusions
We demonstrate that dynamic changes in the level of O-GlcNAc at S284 within FoxO3a could impact on tumorigenesis of PDAC. It is also proved that O-GlcNAcylation of FoxO3a seems very important in cell proliferation that is partly controlled by p21. It may be plausible to propose that the O-GlcNAc at FoxO3a can serve as a signature of PDAC progression as well as a potential drug target for PDAC treatment.

Keywords
FoxO3a, O-GlcNAcylation, p21, pancreatic cancer, PANC-1 cells

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Prioritizing Popular Proteins in Liver Cancer. Remodelling of One Carbon Metabolism.

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Background
Primary liver cancer (HCC) is recognized as the fifth most common neoplasm and the second leading cause of cancer death worldwide. In this study we report a list of popular proteins that have been highly co-cited with the expression "liver cancer". Interestingly, several enzymes highlight the known metabolic remodelling of liver cancer cells, four among them participating in one carbon metabolism. This pathway is central for the maintenance of differentiated hepatocytes as it is considered the connection between intermediate metabolism and epigenetic regulation. We have then designed a targeted SRM method to follow up the one-carbon metabolism adaptation in mouse HCC and in several models of liver diseased conditions at risk of HCC.

Methods
The popular protein list for "liver cancer" was configured with PubPular 2.1 (https://heart.shinyapps.io/PubPular/) SRM assays were set up for 12 one-carbon metabolism enzymes. The method has been tested in different tissues and cell lines with optimal results. Experimental models liver damage and HCC were set to define coordinated variations on steady state levels of one carbon metabolism enzymes.

Results and discussion
The SRM method allowed measurement of MAT1A, MAT2A, MAT2B, GNMT, SAHH, CBS, CGL, BHMT, CHDH, MTAP, SHMT and DYR and revealed a tissue specific expression profile, likely resulting for the specific metabolic requirements of this organ. Pathway remodelling was detected in the liver of mice fed with high fat and choline and methionine deficient diets, upon CCl4 induced liver injury and in liver tumours. The method provides optimal results for formalin-fixed paraffin-embedded liver samples.
Conclusions
Popular protein lists provide valuable information to guide functional studies to better understand human physiology and disease. The method described here allows for the systematic monitorization of one carbon metabolism and may probe its usefulness to asses liver parenchymal cells homeostasis.

Keywords
B/D-HPP, liver cancer, one carbon metabolism, SRM

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Taking up the “neXt50 challenge” using human sperm

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Background:
The present study is a contribution to the “neXt50 challenge”, a coordinated effort across C-HPP teams to identify the 50 most tractable MPs on each chromosome. We report the targeted search of 40 theoretically detectable MPs from chromosomes 2 and 14 (Duek et al., 2016) in the membrane-enriched sperm fraction, together with an update of the total sperm proteome.
Methods:
Pools of human spermatozoa from a total of 15 healthy donors were treated using a Triton X100 fractionation protocol that promotes membrane proteins enrichment. A targeted MS-based strategy consisting in the development of LC-PRM assays (with heavy labeled synthetic peptides) targeting 102 proteotypic peptides of the 40 selected MPs was used. Further validation by immunohistochemistry on human testes sections and cytochemistry on sperm smears was performed when antibodies were available from HPA. Besides, shotgun LC-MS/MS analyses were performed and validated according to HPP data interpretation guidelines (V. 2.1.0).
Results and Discussion:
Thanks to our membrane-enriched sample preparation and targeted LC-PRM assays, 13 of the 40 selected MPs were validated with 2 or more peptides and 5 with 1 peptide. The expression pattern for some of these proteins was studied by immuno-histochemistry and -cytochemistry on whole testis sections and sperm smears. We additionally propose an update of the total sperm proteome combining LC-MS/MS on the present samples with previous data (Jumeau et al., 2015; Vandenbrouck et al., 2016).
Conclusion: Deep analysis of human sperm still allows the validation of MPs and therefore contributes to the C-HPP worldwide effort. Besides, we anticipate that our results should be of interest for the community of reproductive biology for an in-depth analysis of these MPs as potential new candidates in the context of human idiopathic infertilities.

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SWATH® Acquisition - a Unique Approach for Untargeted Metabolomics Applications

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Background: SWATH® acquisition, a data independent acquisition (DIA) workflow, is well adopted in quantitative discovery proteomics, but not commonly used in discovery metabolomics. SWATH® acquisition combines the benefits of high quality MS/MS quantitation (like in targeted MRM-based workflows) with the coverage obtained from untargeted DDA workflows for metabolite identification. Here we describe the improvements in metabolite coverage using SWATH® acquisition without sacrificing quantitation.

Method: SWATH® acquisition experiments on a TripleTOF® System were compared with DDA experiments and processed in a variety of way to characterize the use of these workflows for metabolomics.

Conclusions: Results obtained here demonstrated a significant improvement of metabolites identified at the MS/MS level by using SWATH® with variable windows in comparison with fixed SWATH® windows in all analyzed matrices. Also we were able to identify up to 45% more metabolites from the spectral library when using SWATH® acquisition (30 variable windows) than by using DDA. More confident MS/MS based identifications then led to more quantifiable metabolites in a metabolite expression experiment. Spiked experiments into matrix samples of heavy labeled metabolites highlighted ten times higher sensitivity (signal-to-noise) using the MS/MS ion to quantitate versus the traditional MS1 approach, thus demonstrating the specificity of SWATH® acquisition vs. more traditional data dependent approaches.

Because of the comprehensive, non-stochastic nature of SWATH® acquisition, more identifications and therefore quantitative information is obtained on metabolites compared to the DDA approach which at the end allows better understanding of the biology.

Keywords: SWATH, metabolomics

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The SOMAscan® assay and SOMAmer® reagents: Characterization and utility of novel biomarker discovery tools

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Background
"Slow Off-Rate Modified Aptamer" (SOMAmer) reagents are novel affinity binders made from single-stranded DNA engineered with amino-acid like side chains. These reagents combine the best properties of antibodies and aptamers – high affinity to thousands of proteins, reproducibly made synthetically. The hydrophobic nature of these binding interactions results in exquisite shape complementarity between reagents and protein targets. SOMAmer reagents have been proven effective in biomarker discovery, diagnostic products, and research tools.
Methods
SomaLogic has developed a proteomic assay called the SOMAscan assay for biomarker discovery that transforms protein concentrations in a biological sample into a corresponding DNA signature that can be measured using any DNA quantification technology. The commercially available SOMAscan assay measures over 1,300 human proteins simultaneously in biological samples, and has been used to find candidate biomarkers in applications from cardiovascular disease to rare genetic disorders to oncology. In order to align with the Global Biological Standards Institute's initiatives on improving the quality of life science reagents, we have begun a comprehensive campaign of SOMAmer reagent characterization using several approaches.
Results
To improve transparency, details of the commercially-available protein constructs used to select SOMAmer reagents are disclosed online. When possible, binding of SOMAmer reagents to human proteins that are closely related to the selection targets has been evaluated. Peptide mass fingerprinting has been used to confirm enrichment of the endogenous protein analyte from human plasma for 123 SOMAmer reagents thus far. Finally, depletion with an orthogonal antibody to the same target has also been used to demonstrate signal specificity in the SOMAscan assay.
Conclusions
SOMAmer reagents have enabled broad proteomic profiling of thousands of proteins in a high throughput manner. Additional characterization of the individual SOMAmer reagents comprising the assay provides valuable insight into the interpretation of SOMAscan results.
Keywords
affinity reagent; biomarker discovery

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Platelet Extracellular Vesicles in Human Plasma contain Biomarkers suited for early Diagnostics of Alzheimer's Disease

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Background: Alzheimer dementia (AD) affects about 6% of the general population over 65. Few of AD-patients are affected because of known gene mutations in the APP-protein or the ß-or γ-secretase triggering an early onset of the disease. For such cases it could be shown that the first biomarker changes can be detected about 25 years before the manifestation of the clinical symptoms of dementia. The majority of the AD-patients belong to the group of the so called "sporadic" type or late onset AD; however, many of them carry gene allele variants like apoE4, apoJ, PICALM or CR1 which are found to be risk factors for getting AD later in life.

Methods: In a multi-omics approach we analyzed distinct subfractions of extracellular vesicles in the blood of healthy volunteers, which are produced by senescent platelets and other cells in the circulation. These were analyzed by Nanoparticle Tracking Analysis, Flow Cytometry, proteomic/lipidomic mass spectrometry, miRNA-microarray profiling and deep sequencing.

Results: PL-EVs showed overlapping particle mean sizes of 180-260 nm, but differed significantly in composition. Less dense, intermediate and dense PL-EVs, respectively, are enriched in lipidomic and proteomic markers for plasma membranes, intracellular membranes/platelet granules and mitochondria. Alpha-synuclein (81% of total expression) accumulated in F1-F2, amyloid beta precursor protein in F3-F4 (84%) and ApoE (88%) and ApoJ (92%) in F3-5. Compared to platelets, PL-EVs are enriched in neurological disease-relevant miRNAs. Oxidized cholesterols and lipids are enriched in F4.

Conclusions: These biomarker candidates may be useful for early diagnosis and monitoring of Alzheimer disease years before the clinical symptoms are arising. This may become a starting point for the development of therapeutical interventions.

Keywords: extracellular vesicles, platelets, AD, multi-omics, precision medicine

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14-3-3 σ/Stratifin as candidate biomarker for smokeless tobacco associated Oral Squamous Cell Carcinoma in Pakistan

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Oral squamous cell carcinoma (OSCC) is the most common malignancy in the head and neck with an annual occurrence of 500,000 worldwide. Higher incidence and mortality rates are observed in developing countries especially Pakistan and India. The major contributing factors to OSCC in Pakistan include several forms of smokeless tobaccos (SLTs) such as betel quid, areca nut, gutka, and naswar that specifically have been reported as unique SLTs in this region. In addition, the most common malignancy sub-site in the Pakistani population is buccal mucosa unlike other populations where tongue is the most common sub-site. Early stage diagnosis plays crucial role in the identification and treatment of OSCC and new biomarkers are needed. Comparative proteomic analysis of OSCC tissue and adjacent normal mucosa was undertaken with samples being subjected to two-dimensional gel electrophoresis and differentially expressed proteins identified by LC-MS/MS (Q-Exactive). Seventeen proteins were significantly altered between normal and tumour mucosa including 14-3-3 σ/Stratifin which is an adaptor protein associated with p53-dependent apoptotic regulation. The expression of this protein was verified using immunohistochemistry (IHC) and Western blot analysis both of which confirmed over-expression of 14-3-3 σ in OSCC tissue. These results agree with previous studies conducted on samples from other malignancy sub-sites and from other SLTs/risk factors. The role 14-3-3 σ/Stratifin of as a potential biomarker in association with sub-site location and exposure to various risk factors requires further detailed investigation. Notably, probing the mechanisms by which 14-3-3 σ/Stratifin and p53 may regulate the interplay between cell proliferation and apoptosis during OSCC would be of interest.

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In-Depth Characterization Of Intact Protein Standards Using Top Down Mass Spectrometry With Multiple MSMS Strategies

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Background
Complete and accurate characterization of intact proteins by mass spectrometry is possible today thanks to the latest technological development. We developed a standard mixture for LCMS quality control and method development. Here we present the top-down analysis of this mixture using various fragmentation techniques and MSn capabilities available on a modified Orbitrap Fusion Lumos Tribrid.

Methods
We performed direct infusion experiments and LC-MS experiments using a Pierce intact protein standard mix ranging from ~9 kDa to ~70kDa MW on an Orbitrap Fusion Lumos Tribrid MS modified with a 213 nm UVPD source and coupled with a Vanquish UHPLC system. Intact proteins were separated using 2.1 mm by 50 mm Acclaim columns with a 4 µm particle size. We performed CID, HCD, ETD, EThcD and UVPD fragmentation MSn experiments for structural investigation. Intact Protein spectra were deconvoluted with ReSpect or Xtract in Biopharma Finder 2.0. MS/MS spectra were analyzed with ProSightPC 4.1 and the ProSightPD node in Proteome Discoverer 2.1.

Results and Conclusions
Optimal dissociation parameters were determined as a function of ProSight P score. It was determined, for example, that UVPD reaction times spanned from 15 to 50 ms for proteins ranging from 9 – 68 kD. ETD reaction times ranged from 20 to 10 ms for proteins ranging 9 – 68 kD To test consistency between infusion and LCMS, these optimized settings were translated to LCMS methods. These data not only confirms the translatability of these results and the value of these standards to optimize conditions during MS method development, but also intact protein LC method development and quality control. We will present here the range of optimized values in each fragmentation mode for proteins across the MW range, and discuss theoretical reasoning behind these experimentally determined settings.

Keywords
Intact protein standard, Top down proteomics, Fusion Lumos Tribrid

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Deciphering interconnected activities of matrix metalloproteinases in the healing skin wound by targeted degradomics

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Matrix metalloproteinases (MMPs) contribute to skin homeostasis, cutaneous wound repair, and skin carcinogenesis. Thereby, they do not only remodel the extracellular matrix, but they also play pivotal roles in immune cell recruitment, angiogenesis and epithelial cell proliferation. At the wound edge, MMPs and tissue inhibitors of metalloproteinases (TIMPs) are secreted by migrating keratinocytes and fibroblasts and form an interdependent zymogen activation network that has been poorly understood. In this project, we apply targeted proteomics to map this MMP activation network and its interconnections on the molecular, cellular and tissue level.
By using recombinant mouse proMMPs 2, 3, 7, 8, 9, 10, and 13 as well as TIMPs 1, 2, and 4, we have developed parallel reaction monitoring (PRM) assays for detection of these proteases and their endogenous inhibitors in complex biological matrices with very high specificity and sensitivity. The power of these assays was uniquely increased by including discriminative features for latent and active MMPs that allow monitoring zymogen removal. Next, we exploited our newly established PRM assays to assess pairwise co-activation of MMPs and generated a first model of interconnected MMP activation at the molecular level. In agreement with previous gel-based studies, we observed activation of MMPs 7, 8, and 13 by MMPs 3 and 10, confirming their function as 'activator' MMPs in MMP activation networks.
Harnessing the power of targeted proteomics, we have built a framework for the detection of endogenous MMP activity that we will apply to study their interdependent activation in cell secretomes from keratinocytes and fibroblasts as well as in mouse wound epidermis and skin papilloma. Ultimately, the delineation of the MMP activation network by targeted proteomics will elucidate how its components temporally and spatially shape the proteome at epithelial-mesenchymal interfaces in conditions of controlled and uncontrolled cell proliferation.

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Prognostic role of molecular forms of B-type natriuretic peptide in cardiovascular disease

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Background
B-type natriuretic peptide (BNP), a clinical biomarker for cardiovascular diseases (CVD), is secreted by cardiac tissue in response to neurohormonal stresses and exhibits cardioprotective properties. However, BNP, a 32-amino acid peptide, is processed in circulation by peptidases to produce molecular forms. Clinical BNP measurements associate with prognosis in CVD patients, but associations with molecular forms are not known. Therefore, we performed multiple analyses to investigate the prognostic role of three major circulating BNP molecular forms (5-32, 4-32 and 3-32) on risk prediction for patients with acute hospitalisations of heart failure (HF) and in myocardial infarction (MI).

Methods
BNP molecular forms were measured in plasma using an immuno-capture method followed by MALDI-ToF analysis. Circulating levels were investigated for associations with adverse events and compared to a common clinical measurement of BNP, through circulating levels of N-terminal pro-BNP (NT-proBNP).

Results
BNP molecular forms independently predicted adverse events in patients with acute hospitalisations of HF (n=904) and MI (n=1078). Prognostic ability was comparable with conventional BNP measurements for mortality at 6 months and 1 year (P=0.002-0.032 vs. P=0.014-0.039, respectively) in HF, and at 2 years in MI (P=0.003-0.027 vs. P<0.001-0.040, respectively). Of the molecular forms, BNP 5-32 showed superior prediction qualities for all endpoints, indicating its utility in prognosis for cardiovascular hospitalisations. Survival analyses indicated a stepped increase in risk of adverse events when levels of molecular forms were stratified by tertiles (p≤0.004).

Conclusions
In the first clinical studies to date, we demonstrate that BNP molecular forms provide beneficial prognostic information for outcome in CVD patients in a comparative or superior manner to current clinical protocols. In the personalised medicine era, measurement of specific BNP forms may provide additive information in cardiovascular conditions and more correctly define risk stratification for acute CVD.

Keywords
B-type natriuretic peptide molecular forms, prognosis, cardiovascular disease, MALDI-ToF-MS

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Stratifying asthma disease in the U-BIOPRED project: large-scale MSE and TDA to define sub-phenotypes

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As part of the pan-European U-BIOPRED consortium, we have utilised state of the art 'omics technologies in combination with systems biology approaches to define severe asthma into molecular sub-phenotypes based upon ‘omic fingerprints and their integration into molecular handprints. Asthma is not a single disease, but has several subtypes (phenotypes), for which the molecular basis of this diversity is poorly understood. Stratification of the disease will enable targeted treatment of patients with asthma and the identification of factors that influence both its severity and its responsiveness to treatment.
We present results from a large-scale quantitative proteomic analysis of 270 induced sputum samples from asthma and healthy study participants using label-free quantitative data independent mass spectrometry, LC-MSE with Hi-3 quantitation. Data were pre-processed and cleaned using a custom in-house developed pipeline. 5574 sputum proteins were identified and quantified. Proteotypes of asthma were identified using topological data analysis (TDA) and standard statistics, in combination with clinical meta data for descriptions of the phenotypes. We present 10 asthma proteotype fingerprints and their associated phenotypes, stratifying asthma disease subtypes and identifying possible routes for disease progression. We present biomarkers of the asthma subtypes, identified by logistic regression using predefined training and verification datasets within the patient sample set. This represents a major advance in the understanding of asthma subtypes; molecular mechanisms, diagnosis and personalised patient treatment.

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UbiBrowser: an integrated bioinformatics platform for investigating human ubiquitin ligase (E3) - substrate interaction network

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The ubiquitination mediated by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) cascade is crucial to protein degradation, transcription regulation and cell signaling in eukaryotic cells. The high specificity of ubiquitination is regulated by the interaction between E3 ubiquitin ligases and their target substrates. Unfortunately, the landscape of human E3-substrate network has not been systematically uncovered. Therefore, there is an urgent need to develop a high-throughput and efficient strategy to identify the E3-substrate interaction. To address this challenge, we developed a computational model based on multiple types of heterogeneous biological evidence to investigate the human E3-substrate interactions. Furthermore, we provided UbiBrowser as an integrated bioinformatics platform to predict and present the proteome-wide human E3-substrate interaction network (http://ubibrowser.ncpsb.org).

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Enhancing protein information by integrating genomics and proteomics in UniProt Reference sets

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UniProt provides a broad range of Reference protein data sets for a large number of species, specifically tailored for an effective coverage of sequence space while maintaining a high quality level of sequence annotations and mappings to the genomics and proteomics information.

Mappings to genomics data allows protein scientists to explore the wealth of genomics including variations relevant to disease. Integrating proteomics experiments provides supporting evidences for the existence of specific isoforms, variants and posttranslational modifications thus enhancing functional protein annotations.

For many years UniProt has been working with the proteomics community in completing and enhancing annotations in order to provide quality Reference data sets to the broader scientific community.

With respect to publicly available bottom-up proteomics data from MS proteomics repositories, UniProt provides mappings to its Reference proteomes via the website (e.g. http://www.uniprot.org/uniprot/Q8NI35#showFeaturesViewer), the FTP site (ftp://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/proteomics_mapping/) and programmatically by means of a new RESTful API (https://www.ebi.ac.uk/proteins/api/doc/) which also provides many additional types of data such as genomic coordinates.

The collaborating MS proteomics repositories have also been cross-referenced from within UniProt.

We currently have 17,054 human reference canonical proteins and 58,865 additional human isoforms mapped to proteomics experiments from EPD (https://www.peptracker.com/epd/), MaxQB (http://maxqb.biochem.mpg.de/mxdb/) and PeptideAtlas (http://www.peptideatlas.org/).

Collaborations to add additional data providers for the mappings are ongoing also in order to expand the covered species. Some collaborations are aimed at global reprocessing of PRIDE and some have a specific focus on posttranslational modifications related studies/data sets.

One collaboration is with the Consortium for Top Down Proteomics (CTDP, http://www.topdownproteomics.org) which is already cross-referenced from within UniProt.
Data from the CTDP repository is used by UniProt for the development of a pipeline to annotate back the UniProt entries and publicly provide the corresponding mappings.
CTDP data includes isoform-specific and variant-specific information for whole proteoforms also bearing PTMs.

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A two-step PRM strategy reveals a new biomarker signature of multiple sclerosis in cerebrospinal fluid

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Background
Multiple sclerosis (MS) is an inflammatory disease characterized by an initial demyelinating event (CIS), followed by remission periods and relapses occurring at irregular intervals. Clinical symptoms and brain imaging allow diagnosis with a good sensitivity. However, there is still a need for prognostic biomarkers of conversion to MS and disease progression after a CIS. Using quantitative proteomics, we previously identified ~ 60 candidate MS biomarkers exhibiting differences in abundance in the cerebrospinal fluid (CSF) from patients with MS and symptomatic controls or from patients with rapid (<1 year) and slow (>2 years) conversion to MS after a CIS. These proteins were combined with previously described biomarker candidates to generate a list of 87 CSF proteins that were quantified by Parallel Reaction Monitoring (PRM) in different patient cohorts.
Methods
We implemented a two-step strategy consisting in i) monitoring all selected candidate biomarkers (226 peptides corresponding to the 87 proteins) in CSF samples from symptomatic controls and MS patients at different disease stages (n=60); ii) verifying biomarkers that passed this qualification step (16 peptides corresponding to 12 proteins) in a new PRM assay using shorter gradient and high-purity, heavy isotope-labelled peptides (AQUA Ultimate, ThermoFisher), in a larger cohort (n=189) comprising patients with MS at different disease stages or with other inflammatory or non-inflammatory neurological disorders.
Results
These studies identified a combination of five biomarkers allowing i) positive diagnosis of MS at any disease stage, ii) discrimination between MS and the other inflammatory or non inflammatory neurological diseases and iii) prognosis of conversion to MS for patients with radiologically-isolated syndrome, a presymptomatic form of MS.
Conclusion
The identified biomarker signature may not only improve MS diagnosis and prognosis but also reveal new mechanisms underlying MS pathogenesis and new therapeutic targets for its management.
Keywords
Multiple sclerosis, biomarker, cerebrospinal fluid, PRM

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Robust, Multiplexed ImmunoMRM Assay for Relative Quantitation of Phosphopeptides in DNA Damage Response (DDR) Pathway

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Background:
Robust quantitative tools for studying DDR pathway are in high demand due to the importance of this pathway in cancer research. Current methods using antibody detection such as ELISA or Western Blots are sensitive and high-throughput but suffer from potential interferences or lack of multiplexing capability. Immuno-MRM assays rely on antibodies for enrichment but use mass spectrometry detection for high peptide specificity. Coupled with the ease of multiplexing and internal standard creation, Immuno-MRM provides a great strategy for quantitative protein/peptide assays. However lack of commercially available reagents make these assays inaccessible to the majority of researchers.

Method:
Here, we describe the key steps involved in developing a full Immuno-MRM kit for quantifying key phosphopeptides in the DDR pathway (47 peptides in a single multiplexed assay) with the goal of making this assay more broadly available to researchers.

Conclusions:
The total workflow from sample preparation to LC-MS/MS analysis and data processing has been extensively optimized and integrated with the explicit goal of removing key user pain points. First, a full reagent kit for ImmunoMRM sample preparation has been developed; each individual component from digestion reagents to capture antibodies has been extensively optimized for good performance and stability in kit format. Studies were performed to simplify and validate performance of each protocol step including antibody-bead conjugation, digestion, immuno-capture efficiency and recovery. In addition, the LC-MRM conditions have been adapted for microflow LC and the data processing pipeline has been established to further simplify the full workflow. Robustness of the assay was verified by analyzing multiple biological samples across multiple laboratories. This validated, multiplexed ImmunoMRM assay for the DDR pathway has been demonstrated to be highly robust and easily adaptable into multiple research labs for targeted quantitation of key phosphopeptides from important proteins in the DDR pathway.

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Targeted Proteomics of T helper Cell Transcription Factors and Lineage Specific Markers

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BACKGROUND: T helper cells act as orchestrators for the response and regulation of the immune system. However, imbalances in their differentiation and presentation are associated with autoimmune diseases. An understanding of the early events that drive differentiation has been the focus of a body of research and is important in determining the aetiologies of such diseases as asthma and type 1 diabetes.
Biological discoveries necessitate validations, typically by Western blot of the target together with known cell lineage markers and loading controls. As an alternative multiplex approach we have developed targeted mass spectrometry assays to simultaneously determine Th cell lineage markers with the aim to overcome the typical problems encountered with antibodies.

METHODS: Th1, Th2, Th17 and iTreg cells were cultured form cord blood CD4+ cells and prepared for proteomic analysis with a filter assisted sample preparation protocol. Heavy labelled proteotypic peptides were synthesized for key transcription factors of the cell lines studied (i.e. TBX21, STAT6, STAT3, RORγ, and FOXP3) together with other targets of interest and loading controls. In total 500 peptides from 43 proteins were evaluated. The synthetic peptides were analysed using a Q Exactive Orbitrap mass spectrometer and methods developed for targeted analysis with the same instrument by Parallel-Reaction Monitoring (PRM) and a triple quadrupole instrument by Selected Reaction Monitoring (SRM). Cell lysates were spiked with the synthetic peptides and the detection and temporal dynamics of the selected proteins evaluated

RESULTS: Native peptides and their analogues were detected for the targeted proteins in the Th cell subtypes. Moreover, the targets could be determined in single analysis with good reproducibility and throughput with high sensitivity and selectivity by both SRM and PRM.

CONCLUSIONS: PRM and SRM enabled targeted detection and validation of Th cell markers indicating their utility for validation and routine QC

Keywords: Th-cells, MS, PRM, SRM

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Elucidating the secondary cell wall cellulose synthase complex stoichiometry by Multiple Reaction Monitoring

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Cellulose is the most abundant renewable polymer on Earth and a major component of the plant cell wall. Cellulose is produced as cellulose microfibrils(CMF) by a large plasma membrane protein complexes called cellulose synthases complex (CSC). CSC has been observed in higher plant to have a six fold symmetry and it is widely accepted that 3 different CESAs are required (CESA 4, CESA 7 and CESA 8) for the CSC of secondary cell wall(1). But the actual ratio of the CESA isoforms within the CSCs remains unresolved. The ratio of CSC for Arabidopsis has been determined to be 1:1:1 though quantitative immunoblotting using isoform specific antibodies previously(2). However, it is not known whether this Arabidopsis CESA stoichiometry constrained CSC model applies to all plants or are there differences between species and possibly even tissues and cell type. In this study, In this study, we used multiple reaction monitoring to quantify the absolute concentrations of each CESAs for the secondary cell wall for Arabidopsis, Spruce and Aspen. 27 peptides were selected from the secondary cell wall CESAs and the CSC stoichiometry were found to be different in Aspen from that in Arabidopsis and Spruce.We have also found that CESA 8 is significantly upregulated for Aspen tension wood and the ratio also changed, which suggest that the cellulose microfibril structure is influenced by the number and type of CESAs present.

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Antigen microarrays identify autoantibodies as potential predictors of response to anti-TNFα therapies in rheumatoid arthritis

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Background
The inhibitors of tumour necrosis factor α (TNFα) have improved the treatment of Rheumatoid Arthritis (RA), the most common rheumatic autoimmune disease. However, 10-40% of RA patients do not respond to anti-TNF therapies. Here, we aimed to identify serum autoantibodies that could predict the response to anti-TNF agents in RA.
Methods
Antigen microarrays built on protein fragments within Human Protein Atlas were used in a two-stage strategy for undirected proteomic profiling the IgG and IgA repertoires of baseline serum from 56 RA patients treated with 4 different anti-TNFα agents and classified after treatment as responders (R, n=33), moderate responders (MR, n=13) and nonresponders (NR, n=10). As a first discovery phase, a sample pool from each group of study (R, MR and NR) was screened on a planar array containing 42000 protein fragments. A total of 161 antigens were selected based on a sample pool-specific intensity threshold for a second phase performed on a suspension bead array platform, where the 56 individual serum samples were assayed. The reactivity in each sample was determined by exceeding the median signal over all antigens plus 20x median absolute deviation.
Results
IgG autoantibodies towards two antigens, PRR16 and ZNF618, showed a significantly different ($P<0.05$) prevalence between all R and MR compared to NR. Moreover, all NR showed IgA reactivity to the antigen SLC39A2.
Interestingly, in patients treated with one specific anti-TNFα (Infliximab), all NR (n=6) showed IgG autoantibodies against PRR16, whereas none MR (n=5) and only 2 out of 13 R displayed IgG reactivity to this antigen. Furthermore, 4 out of 6 NR showed IgA reactivity to PRR16, but it was not observed in any of R.
Conclusions
Upon further validation, our findings provide information that could help to predict the response to anti-TNFα therapy in RA.
Keywords
Rheumatoid arthritis, Autoantibodies, Personalized therapy

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Temporally- & Spatially-Resolved Microproteomics and MALDI MSI Lipidomic Analysis for Traumatic Brain Injury Biomarker Identification

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Background=Traumatic brain injury (TBI) represents a health concern worldwide and is a consequence of direct mechanical damage to brain tissue leading to axonal disruption concomitant widespread neural dysfunction. Several studies have assessed potential TBI biomarkers, including GFAP, MAP2, UCH-L1 and αII-spectrin. In this work, we investigated the impact of TBI at the injury site and surrounding tissue, in order to connect TBI microenvironment protein dynamics along with the underlying biological processes associated with the location and proximity of TBI lesion.

Methods=Spatially-resolved microproteomics and MALDI MS Imaging for lipids were used to examine the injury site along with the adjacent regions of an experimental TBI rat model of mild/moderate controlled cortical impact (CCI) injury. Direct on-tissue micro-digestion followed by micoextraction from 0.25-1 mm2 surface areas were subjected to LC-MS & MS/MS analysis using HR-MS. We focus on studying and identifying differential proteins and lipids altered in a spatial (three different brain areas of the cortical impacted section: prior injury, post injury, and injury area in a coronal view of the brain) and temporal manner (1, 3, 7, and 10 days post injury).

Results=An average of 1000 proteins were identified per 1 mm2 of digested and extracted area. At day 3 post injury, proteins specific to injury area were involved in 123 biological pathways mainly participating in inflammation and ROS generation. In addition, MALDI MSI lipid imaging allowed to visualize the presence of lesion specific small molecular weight lipids with possible role in the recruitment, activation and modulation of the neuroimmune system.

Conclusion=In this work, we characterized the “Lipo-Proteomic” profile of the injury lesion and adjacent areas using an advanced neuroproteomics platform. This work will help in the better understanding and the assessment of lesion-specific proteomic and lipidomic biomarkers that can be utilized for targeted therapy.

Keywords=TBI Biomarkers, Microproteomics, MALDI MSI lipidomic

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Building ProteomeTools based on a complete synthetic human proteome

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Background

The ProteomeTools project aims to synthesize 1.4 M million tryptic and non-tryptic peptides as research reagents to cover all human proteins, annotated isoforms, a number of pathological variants and post-translational modifications as well as other relevant biology, like HLA derived peptides. Multimodal LC-MS/MS analysis of these peptides will provide the analytical basis for understanding the true composition of human proteomes, facilitate the design of multiplexed assays as well as the development of better software tools for proteome research.

Methods

Peptides were individually synthesized following the solid phase synthesis strategy on membranes. Pools of 1000 peptides were analysed by LC-MS/MS using an Orbitrap Fusion Lumos ETD mass spectrometer. Spectra were generated using a total of eleven different fragmentation modes and collision energies. RAW data analysis was performed using MaxQuant 1.5.3.30, Mascot 2.6 or SequestHT, data analysis was performed using custom R scripts.

Results

To date, we synthesized and analyzed >500,000 tryptic peptides, covering essentially all canonical human genes as annotated in Swissprot as well as annotated isoforms, major PTMs i.e. phosphorylation, acetylation, ubiquitination as well as less studied PTMs e.g. citrullination. We exemplify the verification of experimental LC-MS/MS data using synthetic standards, examine the fragmentation behavior of modified and TMT-labeled peptides and address the transferability of our spectra to other instrument types and chromatographic systems. To outline possible exploitations of the acquired spectra for data analysis we show their application in fragmentation intensity prediction and rescoring of PSMs to increase the number of true positive identifications.

Conclusions

This is the largest systematic effort to cover the human proteome with synthetic standards, including peptides carrying post-translational modifications. We are confident that the spectra and ultimately the molecular and digital tools arising from the ProteomeTools project will become a very valuable resource for the proteomics community.

Keywords

ProteomeTools, spectral library, synthetic peptides

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Somatic MED12 Nonsense Mutation Escapes mRNA Decay and Reveals a Motif Required for Nuclear Entry

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Background
MED12 is a key component of the transcription-regulating Mediator complex. Specific missense and in-frame insertion/deletion mutations in exons 1 and 2 have been identified in uterine leiomyomas, breast tumors, and chronic lymphocytic leukemia.

Methods
Here, we characterize the first MED12 5' end nonsense mutation (c.97G>T, p.E33X), identified in acute lymphoblastic leukemia, using fluorescence microscopy, biochemical methods, and affinity-purification coupled to mass spectrometry (AP-MS).

Results
Our results show that E33X escapes nonsense-mediated mRNA decay (NMD) by using an alternative translation initiation site. The resulting N-terminally truncated protein is unable to enter the nucleus due to the lack of identified nuclear localization signal (NLS). The absence of NLS prevents the mutant MED12 protein to be recognized by importin-α and subsequent loading into the nuclear pore complex.

Conclusions
Due to this mislocalization, all interactions between the MED12 mutant and other Mediator components are lost. Our findings provide new mechanistic insights into the MED12 functions and indicate that somatic nonsense mutations in early exons may avoid NMD.

Keywords
MED12, BioID, nuclear localization signal, AP-MS

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Targeted Detection of Peptides in Complex Matrices combining the MIDAS™ Workflow with the Skyline Software

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Introduction:

The MIDAS™ Workflow leverages the unqiue hybrid nature of the QTRAP® System and enables the MRM triggered acquisition of full scan ion trap MS/MS. This allows the user to predict peptide MRMs, then detect and confirm these in matrix. This workflow has been implemented in Skyline Software to streamline the selection of peptides for targeted assays.

Methods:

The implementation of this workflow is described in detail in the work. From a protein sequence, peptides and MRMs are generated in silico by Skyline and used to build a peptide detection (MIDAS Workflow acquisition method) which is then run on the QTRAP® system on the matrix. Resulting data is imported back into Skyline which analyzes both the MRM data and the MS/MS data to determine which peptides are detected. The MS/MS spectra is then used to further refine MRM transitions for the detected peptides.

Preliminary Data:

Previous work has demonstrated that MRM triggered MS/MS can detect lower level proteins and therefore will be used here as an MRM assay development strategy.
The steps in the Skyline workflow were first prototyped and optimized using a simple protein digest (Beta Galactosidase). The protein sequence was entered, followed by in silico digestion and MRM computation. A set of MIDAS™ Workflow methods were built, then tested on the QTRAP system. Import and review of detection results allows the method settings to be optimized and the Skyline workflow to be streamlined. MS/MS spectra from the MIDAS file is used to build a standalone MIDAS library file which can then be used for assay development similar to a library generated from a library search, but no additional MS acquisition was required. The workflow was next applied to complex matrices (Ecoli, plasma, yeast) to further optimize workflow efficiency. Skyline workflow and instrument settings are described in detail here.

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Establishment of a fully synthetic, mirror-image biological system.

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Background:
Systems Biology has become a key research area in the quest for understanding living cells. What is missing is a complementing artificial experimental system in which the knowledge and models resulting from systemic analyses could be reproduced and studied without the introduction of any biological bias. Our goal is to create a mirror-image synthetic biology: that is, to mimic, entirely independent of nature, a biological system and to re-create it from artificial component parts.

Methods:
Starting from L-nucleotides and D-amino acids – the enantiomeric forms of the respective naturally occurring molecules – we use chemical synthesis to establish such an artificial system.

Results:
Several basic DNA-DNA, DNA-protein and protein-protein interactions as well as the functions of few enzymes have been copied from nature already, using a totally synthetic approach based on L-DNA and D-proteins. Eventually, this should lead to the establishment of a fully synthetic self-replicating system, including D-protein production, and – in the long run – an archetypical model of a cell.

Conclusions:
Next to the establishment of components for artificial biology, there are immediate practical utilities that are pursued. One of them is the production of biomedically active agents, such as non-immunogenic antibody drugs or other binder formats. Also, once a self-replicating system will be established, biological systems could be set-up that allow the evaluation of basically all their components without any possibility of having a contamination by any naturally occurring compound.

Keywords:
D-proteins, enantiomers, chemical synthesis, synthetic biology, enzymatic activity

Acknowledgement: the project consortium MirrorBio is funded by grant given to the partners within the ERASynBio programme.

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Targeted quantitative screening of Chromosome 18 encoded proteome in plasma samples of astronaut candidates.

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Background
Plasma is a valuable biological material which can be easily obtained from human subjects. Large-scale quantification of plasma proteins represents an important task needed for new biomarkers development. In this context, reference concentrations are known only for selected plasma proteins and most of them have been studied by the mean of routine clinical biochemistry. Approximate reference levels have been published for 150 proteins, representing less than 1% of the putative plasma proteome. If some proteins are found in a disease and are suggested to serve as potential biomarkers, one should know the concentration range of such proteins in a healthy person to predict their applicability.
Methods
Plasma samples of 54 healthy male subjects were used in the study. All the persons were approved for space-related simulations and experiments. The measurements were performed using SRM and stable isotope-labeled peptide standards (SIS) added to each individual plasma sample.
Results
This work was aimed at estimating the concentrations of proteins encoded by human chromosome 18 (Chr 18) in plasma samples of 54 healthy male volunteers. These young persons have been certified by the medical evaluation board as healthy subjects ready to space flight training. Over 260 SIS were synthesized to perform the measurements of proteins encoded by Chr 18.
Selected reaction monitoring (SRM) with SIS allowed to estimate the levels of 84 of 276 proteins encoded by Chr 18. These proteins were quantified in whole and depleted plasma samples. Concentration of the proteins detected varied from 10-6 M to 10-11 M. A minor part of the proteins (mostly representing intracellular proteins) was characterized by extremely high inter individual variations.
Conclusions:
The results provide a background for studies of potential biomarker in plasma among proteins encoded by Chr 18.
The work is supported by the RSF grant №16-44-03007.
Keywords: SRM, SIS, plasma

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Phosphoproteomics of non-small-cell lung cancer cells treated with erlotinib reveals drug-resistant signatures and potential targets

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Phosphoproteome is one of key signatures to understand the mode of action and mechanism of drug resistance of kinase inhibitors at the molecular level, pathway level and system level. We developed sensitive and high-throughput phosphoproteome and tyrosine phosphoproteome analysis platform and performed temporal characterization of non-small-cell lung cancer cell lines treated with erlotinib.
We obtained phosphoproteome and phosphotyrosine-proteome profiles of two erlotinib-sensitive cells and four erlotinib-resistant cells treated by erlotinib for 0 h, 6 h and 24 h. We quantified over 12000 phosphorylation sites by phosphoproteome analysis and over 600 phosphorylation sites on tyrosine residue by phosphotyrosine-proteome.
We extracted kinases and other enzymes which are up-regulated in resistant cells and selected 46 inhibitors for drug screening. 24 of 46 inhibitors inhibited cell growth of at least one resistant cell line.
Our sensitive and high-throughput phosphoproteome and tyrosine phosphoproteome analysis platform has a potential to identify pharmacoproteomic markers of drug efficacy as well as candiates of molecular targets to overcome drug resistance.

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Reproducible In-depth Proteome Analysis of Formalin-Fixed Paraffin-Embedded Tissues by Data Independent Acquisition Method

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Background

Over the last century large biobanks of formalin-fixed paraffin-embedded (FFPE) tissue samples have been established for a wide variety of diseases offering a great potential for a better understanding of health and disease. These samples were shown to be potentially accessible to proteomics. But the reversal of the chemical cross linkage often resulted in low sample preparation reproducibility and low number of identifications. Here we present a highly reproducible workflow to analyze FFPE tissue samples and the application in a small lung cancer pilot study.

Method

Slices of three healthy and three lung cancer FFPE tissues (5um) were dewaxed by Xylene and rehydrated by a graded series of ethanol/water. Samples were lysed in SDS containing buffer by repeated sonication and boiling. After tryptic digestion, digests were analyzed by 2h gradient on a Q Exactive HF (Thermo Scientific) by data-independent acquisition (DIA). A spectral library was generated based on high pH reverse phase (HPRP) fractionated samples, analyzed by data dependent acquisition (DDA). DDA data were searched with MaxQuant and DIA data were analyzed in Spectronaut (Biognosys).

Results

Our workflow resulted in the extraction of 50 to 200ug of peptides per 5um FFPE tissue slice. For the targeted analysis of the DIA data, a spectral library was generated comprising 8’000 protein groups and 125’000 peptide precursors. Sample preparation reproducibility was very high (5-10% CV on protein level) compared the biological variance (23% in the healthy and 36% in the cancer cohort). Within the whole experiment 7’000 proteins were quantified. Statistical analysis revealed more than 2’000 proteins with statistical significant differential abundance. Pathway analysis revealed the regulation of cancer specific pathways.

Conclusion

The combination of a highly reproducible sample preparation workflow with in-depth proteomic analysis demonstrated the high potential of FFPE samples for further research.

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Proteome Profiles of Malignant and Non-malignant Human Colon

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Background
Colorectal cancer (CRC) is the 3rd most common cancer worldwide. While the detection and treatment of this disease is a high priority in developed countries, it does not gain the same attention in less developed ones such as Pakistan. Although the population is defined as low risk, cultural barriers often mean that the disease is advanced when detected and the mortality rate is high. The development of a biomarker-based diagnostic would enable greater acceptance of screening programmes for the disease. We have used normal colon and tumour biopsiesfrom non-cancer and cancer patients, respectively, to investigate proteomics changes with disease progression in a single ethnic group.The aim of this pilot study was to identify biomarker candidates associated with advancement of CRC.
Methods
Proteins were extracted from individual colon biopsies, protein determined and pooled to provide 4 samples of defined pathological stage;normal, non-adenomatous polyp, non-metastatic tumour and metastatic tumour. Pooled samples weresubject to trypsin digestion, 4-plexiTRAQ labelling, 2D LC, analysis by Orbitrap Fusion analysis and database searching on Mascot for protein identification.
Results
Database searching resulted in the identification of 2,777 proteins (95% confidence interval threshold, $p<0.05$, Mascot score ≥ 29) with at least 2 signature peptides and associated iTRAQ reporter ion data. Cluster analysis identified proteins that were significantly changed with disease progression. Of the proteins significantly changed in tumour stages compared to benign/normal, 121 and 198 were increased or decreased, respectively, includingestablishedCRC associated biomarkers, such asCEACAM6, MMP-9 andCAV-1
Conclusions
Primary tissues provide a valuable resource for investigating CRC progression that cannot readily be achieved with cell lines and preclinical models. State-of-the-art mass spectrometry enabledthorough analysis of limited biopsy samples.Our results have provided candidate biomarker panel for further investigation of CRC disease progression and development of a diagnostic strategy.
Keywords: colorectal cancer, iTRAQ proteomics, Orbitrap Fusion, biomarkers

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Development of integrated proteomics database: jPOST

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Background

Large amounts of mass spectrometry-based proteomics data are generated from various institutions and different projects. However, the data integration and comparison between projects are difficult, because those institutions and projects employ different analysis workflows and validation methods. To standardize the workflow and integrate the proteomics data, we have initiated the jPOST (Japan ProteOme STandard Repository/Database) project, which consists of the following three developments: raw data repository, standardized re-analysis protocol for raw data, and integrated database. Here, we introduce the feature of our database currently being developed.

Methods

The jPOST database employs the Resource Description Framework (RDF) data model to store peptide/protein identification results. RDF expresses the relationship between resource objects that represented by URIs as universal unique identifiers, and makes it easier to link data from multiple databases and more useful in computational applications. Currently, life science data in various fields such as genomics, transcriptomics, and metabolomics have been published by RDF. The application of the RDF to the database thus facilitates more flexible utilization of outside resources represented in RDF such as EMBL-EBI resources including UniProt, and the NBDC RDF portal.

Results

We are developing the database including various samples not only from human but from a wide range of organisms such as other vertebrates, plants, and bacteria. In this presentation, we will introduce several specialized data viewers for the protein expression in disease tissues and iPS cell lines, for post-translational modification including phosphorylation sites, and for quantification information including absolute quantification based on the MRM assay. Users can refine and compare search results easily on the web browsers.

Conclusions

In the jPOST project, we are also developing the re-analysis protocol for peptide identifications to reduce false positive/negative hits. The database containing re-analyzed high quality data will be published within this year.

Keywords

jPOST, repository, database, RDF

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6Towards a quantitative map of the human proteome: Monitoring of tissue specific proteins during meningitis

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The protein composition of many body fluids is variable, dynamic and contains proteins from surrounding tissues and cells. In some cases, some of these tissue-specific proteins have been shown to increase in abundance in for example blood plasma under certain pathological conditions. Currently, it still remains unclear however to what extent various tissues can populate body fluids at a systemic level under physiological and pathological conditions. Here we demonstrate how a tissue and cell specific spectral library in combination with data independent analysis mass spectrometry (DIA-MS) can enable the monitoring of tissue-specific proteins directly in body fluids such as blood plasma and cerebrospinal fluid. The spectral library contains over 10'000 proteins generated by extensive shotgun MS analysis of 28 tissues from healthy human controls. To enable improved classification of tissue specific proteins, we constructed a quantitative protein tissue distribution map of the 28 tissue types using DIA-MS. In subsequent experiments we used the quantitative protein tissue distribution map to monitor tissue specific proteins in a cohort of 158 cerebrospinal fluid (CSF) samples from patients with meningitis. The results revealed highly distinct tissue specific protein profiles depending on pathogen-type enabling patient classification with high sensitivity and specificity. Most notably, acute bacterial meningitis introduces a strong reduction of brain proteins and elevation of neutrophil proteins observed in both patient samples and in animal models. Further analysis revealed that neutrophil-specific proteins are derived from neutrophil extracellular traps (NETs), produced exclusively in cases of bacterial meningitis. Removal of the NET associated extracellular DNA improved bacterial clearance in vivo, and represents an interesting future opportunity for the development of new treatment modalities for meningitis. Collectively, these results demonstrate how a large-scale protein distribution map of the human proteome supports the quantification of tissue specific proteins in body fluids using DIA-MS.

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A proteomics approach to subclinical atherosclerosis

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Background: Atherosclerosis is a chronic disease that is usually detected at advanced stages or after a cardiovascular event. The analysis of new mediators or therapeutic targets in human samples has been mainly performed on tissues corresponding to advanced stages. Our aim was to identify new mediators or mechanisms involved in the initial stages of human atherosclerosis.
Methods: A multiplexed, quantitative proteomics analysis of aortic tissue homogenates from individuals with subclinical atherosclerosis [fatty streak (n=7) and fibrolipid lession (n=11)] and controls (n=9) was performed on both the media and intima layers. The protein extracts were digested with trypsin and the peptides were isobarically labelled using TMT. Samples were fractionated by cation exchange and cleaned-up before LC-MS/MS analysis. Statistical analysis was performed based on the weighted spectrum, peptide and protein and systems biology triangle models to pinpoint significant changes in protein abundance. Moreover, protein oxidation was analyzed by studying the dynamic behavior of oxidation-sensitive cysteine residues based on our in-house developed FASILOX method.
Results: We observed, at both the media and intima layers, a simultaneous increase of proteins and processes belonging to lipid metabolism, complement activation, clotting activation, acute phase response proteins and serpins. Conversely, a decrease in proteins related to microtubule organization, heat shock proteins and proteins responsible for mRNA processing was also described. Interestingly, we also observed a strong increase of reversible protein oxidation in the media and intima of patients with initial stages of atherosclerosis as compared to controls.
Conclusions: This comprehensive proteomics study has provided mechanistic insight into subclinical atherosclerosis and identified a variety oxidation-sensitive proteins with a potential role in disease progression. These changes has been also observed in the media, suggesting that this layer should be relevant when designing novel strategies to prevent atherosclerosis development.
Keywords: subclinical atherosclerosis, new mediators, reversibleprotein oxidation, lipid metabolism.

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Development of an analytic interface for multiple peptide library search and label-free comparative proteomic analysis

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Background
We previously developed the "Combo-Spec Search" method, which uses manually multiple references and simulated peptide library searching to analyze whole proteome in biological sample. Although this method shows high sensitivity and fast data processing performance, it is cumbersome to manually process large mass spectrometry (MS) data files. To resolve this problem, we now have developed a new analytical interface tool, called "Epsilon-Q" which was designed to automate the Combo-Spec Search process. This software also supports label-free based comparative proteomic analysis by integrating precursor peak intensities.
Methods
This software supports single or multiple peptide library search methods. It can be applied to in-house built or public spectral/peptide libraries search with simulated MS/MS spectra. Following the peptide library search, false-discovery rate (FDR) was individually estimated per each library. After peptide and protein sequencing and FDR estimation, Epsilon-Q applies appropriate threshold to filter out matches and calculate sum of precursor ions peak intensities.
Results
Epsilon-Q support multiple peptide library search. Especially, Combo-Spec search mode shows improved protein sequence coverages over those obtained from protein sequence database search engines. For each protein, Epsilon-Q estimates all peptide abundance ratios in samples and removes outlier to make accurate protein abundance prediction. In overall, it shows improved performance over the label-free based comparative proteomic analysis.
Conclusions
Epsilon-Q supports Combo-Spec search method and estimates protein abundances by extracting ion chromatograms for comparative proteomic analysis. It has a user-friendly graphical interface and demonstrates a good performance in identifying and quantifying proteins by supporting standard MS data formats and spectrum-to-spectrum matching powered by SpectraST. By using this software, user can automatically analyze their own MS data sets by Combo-Spec Search and label-free based protein quantification.
Keywords
Combo-Spec Search, Peptide Spectral Library, Label-free Quantification

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Shedding new light on Spinal Cord injury

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Spinal cord injury (SCI) belongs to currently incurable disorders of the CNS and is accompanied by permanent health consequences-disability. In order to mimic a SCI, a balloon-compressive technique was used at thoracic Th8-9 spinal level in adult rat. Shot-gun proteomic was used to identify proteins in each spinal cord segment-derived conditioned medium along the rostral-caudal axis after SCI with time course. 3D MALDI Imaging, tissue microproteomics were undertaken combined with confocal imaging. In-vitro and in-vivo tests were realized. We established the spatial and temporal events occurring in acute phase after SCI. Caudal segment has clearly been detected as the therapeutic target. We then assessed in a rat SCI model the in vivo impact of a sustained RhoA inhibitor administered in situ via functionalized-alginate scaffold. In order to decipher the underlying molecular mechanisms involved in such a process, an in vitro neuroproteomic-systems biology platform was developed in which the pan-proteomic profile of the dorsal root ganglia (DRG) cell line ND7/23 DRG was assessed in a large array of culture conditions using RhoAi and/or conditioned media obtained from SCI ex-vivo derived spinal cord slices. A fine mapping of the spatio-temporal molecular events of the RhoAi treatment in SCI was performed. The data obtained allow a better understanding of regeneration induced above and below the lesion site. Results notably showed a time-dependent alteration of the transcription factors profile along with the synthesis of growth cone-related factors (receptors, ligands, and signaling pathways) in RhoAi treated DRG cells. Furthermore, we demonstrate the inflammatory response process involvement via immunoglobulins by binding to their C16/CD32 receptors on the DRG cells upon neurite outgrowth initiation and thus modulating the neurite outgrowth process. We then validate our results by an in vivo proteomic studies along the spinal cord segments. Taken together, we shed new light on Spinal Cord injury

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Investigation of 5-Fluorouracil Resistance in Colorectal Cancer

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Background: One of the leading causes of high mortality rate in colorectal cancer (CRC) is acquired resistance to therapeutic treatment. 5-Fluorouracil (5FU), the main postoperative treatment for CRC acts by blocking production of DNA in replicating cells. In order to understand resistance mechanisms at the molecular level, two CRC resistant cell lines were developed by continuous exposure to 5FU. A SILAC approach was used to compare the resistant cell lines with the parent wild-types, and observe molecular changes relating to resistance.
Methods: CRC cell lines (DLD-1 and HT-29) were grown in the presence or absence of 5FU over a period of 10 months to develop stable, resistant sub-clones and equivalent generation controls, respectively. The parental cell lines were each grown in heavy SILAC media and the resistant sub-clones along with short- and long-term generation controls in light SILAC media. Proteins were extracted, matched “heavy” parental and “light” sub-clone or generation controls combined prior to trypsin digestion, 2D LC, Orbitrap Fusion mass spectrometry, protein identification and quantification.
Results: DLD-1 and HT-29 sub-clones were 130- and 3.5-fold more resistant to 5FU than the respective wild type and generation controls. From 3 SILAC experiments each, for DLD-1 and HT-29, an average of 4978 and 4394 unique gene products were identified (confidence threshold $p<0.05$, ≥1 unique peptide, ≥ 2 PSMs), respectively. There were 14 up- or down-regulated proteins common to both resistant cell lines, whereas 197 and 161 were uniquely changed in DLD-1 and HT-29, respectively.
Conclusions: That there were few common proteins reflects the heterogeneity of resistance mechanisms in CRC and the importance of developing more than one cell line model. The greater resistance achieved in DLD-1 (cf. HT-29) revealed protein changes associated with micro-satellite instability, an established phenotypic difference between the two cell lines.
Keywords: colorectal cancer; drug resistance, SILAC proteomics; 5-fluorouracil

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READYBEADS: A new tool to improve robustness and reliability in LC-MS analyses

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Mass spectrometry coupled to Liquid Chromatography (LC-MS) is a powerful tool for the characterization and the quantification of large molecules in complex
biological matrices. To obtain robust analyses that can be transferred, throughout each step of the development of new drugs or during validation of biomarkers,
sample normalization with internal standards are essential. However, getting reproducible internal standard solutions, notably when several molecules are
needed, is complex and requires several validation steps. Indeed, biomolecules might not be stable in solution and might need storage in a compatible container,
at a specific concentration and temperature (1). Moreover, generating dozens or hundreds solutions at a defined concentration is time consuming and leads to
poor reproducibility, especially between operators.
In order to solve these issues, we have developed an innovative technology called READYBEADS. This technology is based on a water soluble biopolymer, that does
not interfere with mass spectrometry analysis, where internal standards are coated and can be released at a controlled concentration.
This innovative technology presents the key property to be stable through time at room temperature, and allows an easy way to prepare reproducible complex
mixture in a minute apart from the operator. This solution allowing ready-to-use customized standards will be presented in this study through the implementation
of READYBEADS for various applications.

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Alternative proteins: the hidden world of potential biomarkers

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A mature mRNA is a molecule presenting three different ORFs displaying several alternative ORFs. Recently, alternative proteins were detected in human cells although such a mechanism was considered as anecdotal in eukaryotes. To increase the number of potentially identified alternative proteins, a combination of multiple algorithms was used using the peptide shaker software. We were able to identify 10364 alternative proteins and 857 phosphorylated alternative proteins. Tissue Top-down microproteomic was firstly performed on 3 brain regions. 156 references proteins from different cellular compartments were characterized. Moreover, 11 Alternative proteins issued from alternative open reading frames (AltORF) were identified and were relied to the brain regions and associated to the function of their reference proteins. Some proteins display specific post-translational modifications profiles or truncation linked the brain regions and their functions. Systemic biology performed on microproteome identified in each region allowed to associate sub-networks with functional physiology of each brain region. Back correlation of the local extracted and identified microproteome with tissue cellular localization was then performed by MALDI mass spectrometry imaging. 40 proteins including 4 Altproteins have been back correlated in MALDI MSI and are in line with their tissue extracted region and physiological function. Then the same strategy was undertaken in order to investigate tumoral tissue microenvironment in ovarian cancer. 289 proteins with post-translational modifications have been isolated from tumoral biospies. Besides the reference proteome, a deeper analysis of proteins issued from alternative ORF, led a total of 14 Altprots. Expression of these AltProteins were investigated by western blot and immunofluorescence experiments with an anti-FLAG and are constitutively co-expressed with their reference proteins. Thus we report for the first time the co-localization of two proteomes: one issued from the kozak code and one from Alternative ORF, so called the Hidden Proteome.

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Proteomic analysis reveals strong secretion of IL-9 by group2 innate lymphoid cells upon IL-33/TL1A co-stimulation

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Background
Group 2 innate lymphoid cells (ILC2s) are key players of innate immunity producing Th2 cytokines IL-5 and IL-13. They are major targets of interleukin-33 (IL-33), which is critically involved in allergic inflammation and asthma. However, the mechanisms of ILC2s' response to the stimulation by IL33 and other activators, such as the TNF-family cytokine TL1A, are not fully understood.
Methods
We used a large-scale mass spectrometry approach to characterize the proteome of ILC2s and its modulation following stimulation. Mouse ILC2s were isolated from lungs, stimulated in-vitro with IL-33 alone or in combination with other cytokines, and analyzed by single-shot nanoLC-MS/MS on a fast-sequencing Orbitrap Fusion mass spectrometer, followed by label-free quantification.
Results
We mined the proteome of ILC2s to a depth of more than 5000 proteins and detected about 200 proteins significantly modulated following IL-33 stimulation, including IL-5 and IL-13 but also several cell surface proteins and transcription factors. Interestingly, our unbiased proteomic approach revealed that stimulation of ILC2s with a combination of IL-33 and TL1A specifically induced a major up-regulation of IL-9, a key driver of chronic and allergic inflammation at mucosal surfaces. Further analysis by flow-cytometry, ELISA and RT-qPCR showed that IL-33 synergizes with TL1A to induce prodigious amounts of IL-9 secretion by ILC2s. The IL-9 production was transient and associated with a phenotypic shift characterized by upregulation of pSTAT5 and downregulation of the ILC2 master transcription factor GATA-3. Finally, in vivo experiments showed that the IL-33/TL1A co-stimulation induces IL-9 positive ILC2s in lungs, and that these cells are potent inducers of allergic airway inflammation.
Conclusion
This is the first proteomic characterization of ILC2s and their response to IL-33 activation. Importantly, we describe for the first time stimulatory conditions that induce the secretion of IL-9 by ILC2s, up to levels significantly higher than previously reported for Th9 cells.

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Quantitative Proteomic Study of Macrophage Proteins interacting with Candida albicans using an ATP Affinity Enrichment

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Background
Macrophages, in collaboration with neutrophils and monocytes, are key cells in the recognition and in innate immune response to the human pathogen Candida albicans. For that, a tight regulation of several signalling pathways is required. ATP binding proteins are involved in processes like inflammation, cytoskeletal rearrangement, stress response and metabolism.
Methods
Here, we optimized a quantitative proteomic approach for the study of human macrophage ATP-binding proteins after interaction with C. albicans cells. For this approach, the human monocytic (THP1) cell-line was used and labelled by SILAC method with switched labelling. Monocytes were differentiated into macrophages by adding PMA. After incubation of the macrophages with C. albicans cells (MOI 1:1) during 3 hours, the protein lysate was enriched in ATP-binding proteins using the ActivX desthiobiotin ATP probes (Thermo Scientific) kit. Then, proteins were reduced, alkylated and in-gel digested using trypsin. Then, samples were analysed by LC-MS/MS using an LTQ-Orbitrap and the fragment ions extracted for a protein database using Mascot.
Results
Overall, 547 non-redundant proteins including 137 ATP-binding proteins were quantified. From them, 22 proteins were more and 37 were less abundant during macrophage interaction with C. albicans. Forty five protein-protein associations were found according to known, predicted and other interactions.
A Gene Ontology term enrichment analysis showed that the differentially more abundant proteins during interaction were involved in RNA splicing and protein synthesis whereas the 37 less abundant proteins were involved in proteolysis events, apoptotic processes and ion transmembrane transport. Western blotting and selected reaction monitoring were used to validate the abundance of three proteins (MAP2K2, PRDX5 and NDKA).
Conclusion
These approaches are crucial for unravelling new proteins involved in the immune response to C. albicans that can be applied in antifungal therapies
This work is supported by FP7-PEOPLE-2013-ITN, IMRESFUN Project.

Keywords: quantitative proteomics; macrophages, Candida albicans, ATP

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Investigation of cardiotoxicity in 3D human cardiac microtissue model

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Background
The leading cause of mortality in cancer patient survivors is cardiovascular disease with an increased eight-fold risk when compared to the general population. Current research into mechanisms of disease and drug safety assessments is carried out using animal models that harbor species variability or stem cell-derived cardiomyocytes, which predominantly represent an immature cell phenotype and so are not representative of those found in the adult heart. Therefore, development of a cell culture system containing mature human cardiomyocytes would be of considerable benefit in cardiovascular research.
Methods
Here, we develop and characterize a readily reproducible, three-dimensional (3D) human cell culture microtissue system that is more emulative of an adult-like human heart. We applied global mass spectrometry, database searching and statistical filtering at the peptide and protein level (<1% FDR), followed by relative quantification based on spectral counts on both early and more mature microtissue to confirm the presence of known and identify previously unannotated proteins differentially regulated upon cardiac maturation. Using this more mature tissue we explore the mechanism of cardioprotection offered by known and novel agents.
Results
Analysis resulted in 1126 differentially expressed protein clusters significantly regulated in human cardiac cell development which showed increased expression of proteins involved in structure and contractile function upon maturation, including α-actinin, RyR2, SERCA and PLN with GO-term enrichment in processes associated with cardiac development. Microstissue showed a dose-dependent decline in cell viability with doxorubicin, which was diminished in the presence of dexrazoxane.
Conclusions
We describe here a readily reproducible, functional 3D human multicellular cardiac system that emulates a more adult-like phenotype. This system provides a viable human in vitro model that is highly applicable for the study of cardiac disease and cardiotoxicity.

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Proteomics Signature in Early Remodeling after Myocardial Ischemia/Reperfusion and the Effects of Experimental Treatments

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Background

Reperfusion alters post-myocardial infarction (MI) repair. Very few systematic studies report the early molecular changes following ischemia/reperfusion (I/R) and their behavior in the presence of protective treatments. Alterations in the remote myocardium have been neglected, disregarding this region's contribution to post-MI heart failure (HF) development. Study's aim is to characterize the protein and reversible cysteine oxidation dynamics in the ischemic and remote myocardium during the first week after MI as well as the effects of experimental conditioning strategies in a clinically-relevant pig model.

Methods

Closed-chest 40min I/R was performed in 25 pigs that were sacrificed at 120min, 24h, day 4 and day 7 post-I/R. Other animals were assigned to: non-reperfused MI, regular 40min I/R with pre-conditioning or post-conditioning strategies. Five control pigs were sacrificed after baseline imaging. Myocardial tissue samples were collected after sacrifice and processed for proteomic analysis. Multiplexed labeling, parallel reaction monitoring, label free and immunoblotting experiments were performed to assess the molecular profiles and validate the results.

Results

At early reperfusion (120min), the ischemic area showed a coordinated upregulation of inflammatory processes, whereas interstitial proteins, angiogenesis and cardio-renal signaling processes increased at later reperfusion (day 4 and 7). In the remote myocardium, unbalanced contractility at 120min and 24h was manifested by transient alterations in contractile and mitochondrial proteins. Pre-conditioning strategy ameliorated the changes associated with early reperfusion, whereas observed alterations in non-reperfusion probably delayed them. While reversible cysteine oxidation did not appear in remote tissue, its maximum was detected at 24h post-MI within the ischemic area, which decreased in the presence of pre-conditioning and non-reperfusion.

Conclusions

Implemented treatments not only allow the molecular profiles recovery from the I/R-related injury and oxidative damage in the ischemic tissue but also influences temporally disengaged energy metabolism and contractile function in the remote heart.

Keywords

myocardial infarction; remodeling; edema

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A quantitative analytical method using multiple reaction monitoring-mass spectrometry for early diagnosis of hepatocellular carcinoma

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Prothrombin induced by vitamin K absence-II (PIVKA-II) is an effective tumor marker for hepatocellular carcinoma (HCC). We have developed a novel targeted mass spectrometric (MS) assay for quantifying PIVKA-II in human serum. The ideal signature peptide was selected to measure PIVKA-II concentrations on a triple quadrupole (QqQ) mass spectrometer, and the chromatography gradient was optimized for the peptide separation to minimize elution interference. Using multiple reaction monitoring-mass spectrometry (MRM-MS), good linearity (R2 = 0.9988) was obtained for PIVKA-II over a range of 3 orders. We achieved a limit of detection (LOD) of 0.45 nM (31.72 ng/mL), a limit of quantification (LOQ) of 0.93 nM (65.31 ng/mL), a lower limit of quantification (LLOQ) of 0.49 nM (34.32 ng/mL), and an upper limit of quantification (ULOQ) of 1000.00 nM (70037.00 ng/mL). The intra-day and inter-day precisions were within ± 14.96%, and the accuracy ranged from 87.66% to 114.29% for QC samples at 4 concentrations. Compared with an established immunoassay, the correlation (R = 0.8335) was good for the measurements of PIVKA-II concentrations. This method was successfully applied to the analysis of clinical samples for normal control (n = 50), chronic hepatitis (n = 50), liver cirrhosis (n = 50), HCC (n = 50), and recovery (n = 50) serum.

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Revealing histone variants by targeted proteomic analysis using the manually curated database MS_HistoneDB

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Background: The crucial roles of histones in most DNA-based processes in eukaryotic cells become more and more elucidated and appreciated. Recent findings suggest that altered expression and replacement of canonical histones by their variants can contribute to carcinogenesis and infertility. Since many family members of histones share high sequence homology, the generation of antibodies that specifically distinguish these members for quantification and functional analysis is a difficult task. Mass spectrometry has emerged as a promising analytical strategy for characterization and functional studies of histone variants. However, their identification from proteomics data remains challenging since no protein database is ideally suited for the analysis of histones and the complex array of mammalian histone variants. We thus created two manually curated proteomics-oriented databases for mouse and human histone variants, respectively. We also developed a targeted proteomic method used to quantify histone variants across different samples.
Methods: Manual sorting of >1700 entries of genes, transcripts and proteins allowed generating two non-redundant lists of 83 mouse and 85 human histones. The current nomenclature was used to annotate the selected entries and they were unified with the "HistoneDB2.0 with Variants" database. This resource is provided in a format that can be directly read by programs used for mass spectrometry data interpretation. We also conducted quantitative proteomic analysis of histone variants during mouse spermatogenesis, in which diverse H2A and H2B variants are expressed. The abundance of H2A and H2B variants was quantified at different stages of maturation.
Results: We developed a robust and efficient method to quantify histone variants. Our investigations allowed the identification of new histone isoform(s) of unknown function, which are expressed in late spermiogenic cells.
Conclusions: The proposed method can be easily transposed to investigate human histones in physiological and pathological contexts, including cancer.

Keywords: Chromatin, Histone variants, Mass spectrometry, Targeted proteomics, Spermatogenesis

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PEPTIDOMIC ANALYSIS OF RAT PLASMA SAMPLES: EFFECTS OF TRANEXAMIC ACID AGAINST PROTEOLYSIS IN HEMORRHAGIC SHOCK

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Background:
Hemorrhagic shock is still one of the main causes of mortality in the intensive care units. Previous studies enlighten the fundamental role of the intestine in shock: according to the so called “Autodigestion hypothesis” the intestinal mucosal barrier, damaged by hypoperfusion, allows digestive enzymes to escape the lumen and reach the systemic circulation leading to a strong inflammatory reaction. A label-free quantitative proteomic investigation, conducted in our laboratory, demonstrated for the first time that plasma displays an increase in peptides possibly generated by serine proteases after hemorrhagic shock (HS), linking proteases to the larger presence of circulating peptides. To further confirm these findings, we adopted the same “peptidomic” approach to verify the possible protective effects of protease inhibitors, such as TXA (tranexamic acid), against the uncontrolled proteolytic activity.
Methods:
We performed a LC-ESI MS/MS analysis to compare the peptidome of plasma samples from HS rats treated with or without TXA. For each animal, 2 plasma samples were collected: at the beginning (BL) and at the end of the experiment (END). The computational analysis was performed with MaxQuant and Perseus software. Peptides were also searched in SATPdb, a database of structurally annotated therapeutic peptides.
Results:
The analysis shows a significantly decreased number of circulating peptides in HS plasma treated with TXA compared to HS plasma. This effect is more evident in the “END” samples. In addition, SATPdb analysis suggests the possible presence of antihypertensive peptides.
Conclusions:
These results allow to quantitatively support the “Autodigestion hypothesis” suggesting an increased proteolytic activity in plasma samples after HS. Further analyses are in progress to better elucidate the possible proteases involved and to test protease inhibitor-based strategies, which could interfere with shock lethal course.
Keywords: peptidomics; hemorrhagic shock; proteolysis; tranexamic acid; mass spectrometry.

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Advanced Centroiding of Low Resolution MS/MS Spectra Provides Higher Confidence Identifications from Proteomics Datasets

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Background

Accurate mass detection via mass spectrometry (MS) is a fundamental aspect for confident identification in proteomics, due to the high complexity of proteomes and the existence of nearly isobaric post-translational modifications. This issue has been addressed by creating more accurate mass analyzers, but such MS is sometimes prohibitive for laboratories, and MS/MS is still mostly performed in ion traps for fast scan rate. We analyzed a novel software, namely PeakInvestigator, that performs peak picking from low resolution MS and MS/MS ion signals using an advanced signal processing methods for peak detection, deconvolution and centroiding. This innovative spectra processing allows for more complete and accurate detection of ion signals, leading to more confident peptide identifications.

Methods

Data presented are HPLC-MS/MS runs performed with an LTQ-Orbitrap Elite (Thermo Scientific) operating in high (MS) and low (MS/MS) resolution mode. We analyzed a (i) synthetic peptide library, including the HUPO sequencing proficiency study; (ii) tryptic digest of a yeast proteome; (iii) human phosphoproteome sample (HeLa).

Results

We compared three software packages for peak picking: PeakInvestigator (Veritomyx), Apex Picking (Proteome Discoverer, Thermo Scientific) and Standard Centroiding (PeakPickerHiRes 2.0.0, OpenMS). PeakInvestigator centroiding consistently yielded higher peptide scores than the other solutions. For the less complex synthetic peptides and yeast lysate samples, all three centroiding packages provided comparable results in number of highly-confident peptide identifications obtained. However, on the more complex phosphorylated human sample, PeakInvestigator increased the number of highly-confident peptide hits by 29% vs. Apex Picking and by 15% vs. Standard Centroiding. In addition, for highly-confident phosphorylation site localizations, PeakInvestigator results were improved by 17% over Apex Picking, and 7% over Standard Centroiding.

Conclusions

We demonstrate how more complete and accurate centroiding delivers a significant positive effect on confidence and number of peptide identifications in complex proteomics datasets.

Keywords

Centroiding, low resolution, PeakInvestigator, spectra processing

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Long Noncoding RNA HOTAIR Promotes Hepatocellular Carcinoma Cell Proliferation by regulating opioid growth factor receptor

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Long non-coding RNA HOX transcript antisense RNA (HOTAIR) is involved in human tumorigenesis and dysregulated in hepatocellular carcinoma (HCC). However, the molecular mechanisms underlying HOTAIR functions in HCC are not well understood. Here, an integrated transcriptomic and quantitative proteomic analysis were employed to systematically explore the regulatory role of HOTAIR in HCC. A total of 673 transcripts and 298 proteins were identified to be dysregulated after HOTAIR inhibition. Bioinformatics studies indicated that the differentially expressed genes (DEGs) and proteins (DEPs) are involved in many biological processes, especially cancer-related signalling pathways. Further functional studies on opioid growth factor receptor (OGFr), a native biological regulator of cell proliferation in HCC, revealed that HOTAIR exerts its effects on cell proliferation, at least in part, through the regulation of OGFr expression. By correlating the omics data with functional studies, the current results provide novel insights into the functional mechanisms of HOTAIR in HCC cells.

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Mass Spectrometry Imaging (MSI) and Laser Micro Dissection for Cancer Diagnostics on Tumour Tissue

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In this presentation we describe the use of Mass Spectrometry Imaging (MSI) using MALDI-TOF MS instrumentation in combination with laser micro dissection and LC-MS/MS analysis as potential tool in cancer diagnostics. We have established a workflow for formalin fixed paraffin embedded (FFPE) tissue from solid tumours to investigate peptide profiles in different areas of the tissue after tryptic digest of the proteins. We can then use these peptide profiles to make prognostic or diagnostic decisions which cannot be made by a pathologist using standard methods like H&E staining or immunohistochemistry. We have shown examples using ovarian and endometrial tumours as proof of principle that this approach is very promising. Additionally, we now have also a workflow for profiling N-glycans from FFPE tissue in order to have an additional layer of information if the peptide profiles are not able to distinguish between clinical relevant cases. The change in N-glycan pattern of cancer cell surfaces compared to normal cell surfaces is well-documented in the literature and Imaging Mass Spectrometry can show those changes spatial resolved in tumour tissue sections.

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Impact of obesity-induced diet on mice brain phospho-proteome

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Background
Obesity is closely associated to several diseases such as type 2 diabetes, cardiovascular disease, neuro-degeneration, biliary disease and certain cancers. Given this fact, it is of importance to assess the role of nutrition in disease prevention as well as its effect in the course of such pathologies. Considering these factors, in this study we addressed the impact of different diets in mice brain development. With the aim of studying the neurological effects of three different types of diet, we explored the mice brains phospho-proteome.
Methods
Obesity was induced in two different groups. Each group composed by 3 males C57BL/6J, fed, respectively, with hyperglycemic (HG) or high-fat diet (HF) for 12 weeks. A control group of 3 males C57BL/6J was fed with a Standard Pellet Diet (SPD). Metabolic parameters were measured before sacrifice and brains were harvested for label free phospho-proteomic analysis. We used mice brains to find differences, if any, in protein phosphorylation mediated by obesity-induced diets.
Results
We detected changes in protein phosphorylation of mice fed with HFD and HGD compared with SPD. Interestingly, obesity seems to be responsible for such changes, independently on the kind of diet (either HFD or HGD). We detected dephosphorylation of proteins involved in neuronal development (i.e. SYNGAP1 and PPP1R9B) and in vesicle trafficking (i.e. SNAP91 and AMPH) and in cytoskeletal functions (i.e. CLASP2 and GSK3B). On the other hand, increased phosphorylation was detected for microtubule proteins (i.e. MAP2 and MAPT).
Conclusions
In the present study we were able to detected changes in phosphorylation of many proteins implicated in key processes for CNS development. Phospho-site analysis reveals the important role played by phosphorylation and the possible connection between obesity-induced diet and brain impairment.
Keywords
Obesity, neuronal impairment, phospho-proteomics, mass spectrometry.

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Complex-centric proteome profiling by SEC-SWATH-MS reveals modularity and dynamics of the cellular proteome

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The cellular proteome is organized into macromolecular complexes that constitute functional modules catalyzing biochemical functions. Mass spectrometric protein correlation profiling along the retention time dimension of chromatographic separation of native complexes has recently emerged as a method for the multiplexed analysis of complexes. Present implementations based on size exclusion chromatography are over determined due to the high number of cellular protein species (5000-10.000) and limited chromatographic peak capacity (10-20), resulting in low specificity of assigned complexes.
To alleviate these limitations we developed a variant protein correlation workflow that is based on a novel, complex centric data analysis strategy and highly reproducible and quantitatively accurate profiling of peptides along consecutive chromatographic fractions. Specifically, the workflow combines high resolution size exclusion chromatography, DIA/SWATH-MS, and multi-level targeted data analysis, extending concepts of peptide-centric analysis of DIA/SWATH-MS data to the level of complex inference in SEC-SWATH-MS. Using prior information on proteome connectivity as input into the targeted data analysis, we detected and quantified more than 800 protein complexes and complex variants from the HEK293 cell line at high specificity. A target-decoy model controls errors among results. The data led to the identification of novel assembly intermediates of the 20S proteasome core particle and of a trimeric subversion of the COP9 Signalosome complex. We further applied the technique to the delineation of protein complex rearrangements in cell cycle progression and shed light on the role of isoform switching and post-translational modification in the functional regulation of proteome rewiring.
In summary, we present a DIA/SWATH-MS-based implementation of protein correlation profiling and the novel, targeted, complex-centric data analysis strategy. The novel method generates a comprehensive, accurate and quantitative view on the cellular protein complex landscape. We expect SEC-SWATH-MS to provide significant new insights into the tempo-spatial organization of the cellular proteome and the functional consequences.

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A primary human cell line for Down syndrome studies

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A primary human cell line for Down syndrome studies

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Human cell models and particularly stem-cell models for Down syndrome (trisomy 21) are, in general, difficult to establish. In the present study, we report the isolation, culture and characterization of multipotent stem cells derived from human exfoliated deciduous teeth (SHED) from an individual with Trisomy 21 (Down syndrome). The isolated Down syndrome SHED (DS-SHED) cells were cultured and analysed regarding morphology, chromosome composition, and gene expression. The proteome of DS-SHEDs was explored by nanoLC-MS/MS bottom-up proteomics in a Dionex Ultimate 3000 nanoHPLC system coupled to an LTQ Velos - Orbitrap Elite mass spectrometer. Results indicated that DS-SHEDs' morphology was typical of mesenchymal cells, demonstrating a fibroblast-like shape with round centrally-located nucleus. Their growth potency was elevated compared to "normal/control" SHEDs. Cytogenetic analysis verified the presence of trisomy 21 in all cells examined. Cells were found to expressed stem cell molecules including Nanog, Oct4, Sox2 and were positive for CD73 and CD90 and the recently suggested potential markers CD166 and CD44. Proteomic analysis of DS-SHEDSs resulted in the identification of 1,945 protein groups (distinct gene products). In silico analysis revealed that 202 genes encoding the differentially expressed proteins were located in chromosome 1, 136 genes located in chromosome 2 etc, and 19 in the chromosome 21. When compared to "normal/control" SHEDs, 585 proteins were overexpressed in DS-SHEDSs and 662 down-regulated. Among differentially overexpressed proteins of DS-SHEDSs, 6 were found located in chromosome 21 (CBR3, PCNT, A4, INAR1, TIAM-1 and OFUT2). In conclusion, we generated primary cells that can provide a useful tool for the study of the gene expression, the cascade of events and the implicated mechanisms in trisomy 21 and potentially in the pathothysiology of Down syndrome.

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Proteomic biomarkers for urothelial carcinoma diagnosis in liquid-based cytology via cross validation with FFPE samples

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Urothelial carcinoma (UC) of the bladder is a disease with high mobility and mortality. Urinary cytologic examination represents the 'gold standards' for surveillance of urothelial carcinoma diagnosis and recurrence. However, the accuracy of urine cytology appears to be associated with considerable variability. Positive results are obtained in 31-72% of patients with bladder cancer. In addition, inter-observer variability varied from 38-65% across institutions. There are a few molecular tests including UroVysionTM, however overall specificity and sensitivity of the test is similar to cytology alone due to pitfalls that cytological appearance is not taken into account. In this study, to identify novel biomarkers in liquid-based method of voided urine cytology, which has technical advantages, we have employed a high-throughput mass spectrometry for in-depth proteomics approaches and an integrative workflow composed of external public databases and tissue proteome for the biomarker discovery being followed by validation of predictive ability of the biomarkers in an independent internal validation set. For discovery set, a total of 20 voided urine cytology samples were enrolled, which consists of 10 cases of primary urothelial carcinoma in urinary bladder and 10 non-tumor controls. Six formalin fixed paraffin embedded (FFPE) urinary bladder tissues from each sample group of four non-tumor histology and four UC samples were included for additional comparative mass spectrometry-based proteomic analysis. For the validation of the selected proteomic biomarkers, an independent internal validation set of 25 UC patients and controls of the non-tumor cohort was enrolled, respectively. The mass spectrometry analysis yielded 4,839 identified protein groups and 2,859 quantified protein groups. After normalization, 112 differentailly expressed proteins were eventually selected. Further cross validation with two separate cohorts revealed 13 proteins commonly identified in three cohorts and two were adopted as new biomarkers for UC diagnosis in independant cytologic patients samples.

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Library and de novo sequencing hybridization: a method for identification of cis-spliced MHC peptides

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Background: Major histocompatibility complex (MHC) molecules play a major role in adaptive immunity by presenting peptides on the surface of cells for recognition by T cells. Recent studies show that a large fraction of MHC-class-I peptides are 'spliced' – i.e. the joining of two distant regions of same protein to produce a non-genomic linear sequence. The greatest barrier to identifying such peptides is the computational resources required to process all permutations of spliced-peptides. We have developed a novel workflow that can be used to rapidly identify such peptides using computationally less-intensive methods.

Methods

MHC–peptide complexes from C1R-HLA-B*57:01 cells were immunoaffinity-purified and analysed on TripleTOF®5600+ SCIEX. Data was firstly searched by de-novo sequencing using Peaks-Studiov7.5 and identifications with 80% accuracy were added back to the human proteome library. This combined database was then searched using ProteinPilot™v5.0 and peptides at 1% FDR cut-off were further processed using an in-house developed algorithm that distinguishes spliced peptides. Synthetic peptides were used to validate peptide identification accuracy.

Results

We have identified 6548 peptides at 1% FDR with 8-12 amino acids (4686 linear and 1862 spliced). A selection of 12 synthetic spliced peptides were analysed by LC-MS/MS, confirming their identity. The majority of sequences were 9-mers (44.5% in the spliced subset). Peptides from both the conventional and spliced sets conformed to the expected HLA-B*57:01 binding motif, with dominant anchor residues at P2 (Ser/Thr) and PΩ (Trp) .

Conclusions

A combination of library search and de-novo sequencing is here proposed for unrestricted identification of spliced MHC-class-I peptides. This method is not limited by the distance between spliced parts, fast and does not need high-performance computational equipment. These results highlight the complexity and diversity of MHC-peptide display and broaden our understanding of T cell immunity, whilst having clear implications in the context of immunotherapeutics such as peptide vaccines

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Integrated omics analyses of oral cancer in Taiwan

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Background
Oral squamous cell carcinoma (OSCC) is a prominent cancer worldwide. In Taiwan, OSCC is a prevalent malignancy that represents the fourth most common cancer in men. Several studies have used genomic, transcriptomic or proteomic approach to investigate the alterations at genomic, transcriptomic or proteomic levels in OSCC but few studies provided integrated-omic analysis in OSCC.
Methods
Here, we conducted the first integrated-omic study with paired samples including 50 pairs for WES, 39 pairs for RNA sequencing and 41 pairs for iTRAQ-based quantitative proteomics analysis. We detected mutations, copy number variations, gene expression alterations, protein abundance alterations and investigated the correlations between these alterations. This was the first oral cancer study in Taiwan to integrate genomics, transcriptomics and proteomics.
Results
We uncovered TP53, FAT1, NOTCH1, PIK3CA, CDKN2A and HRAS as frequently mutated genes, and EGFR, FGFR1, FADD, FAT1 and CDKN2A as frequently amplified/deleted genes. We found that the mutation signatures are similar to those reported in TCGA-HNSC. Similar to that present in the TCGA transcriptomic data, we also identified activated pathways including cell-matrix interactions, interferon signaling, ECM remodeling from 39-paired RNA sequencing data. The iTRAQ-based quantitative proteomic results of the same set of samples revealed the significant alteration of the above-described pathways. We further investigated these copy number variation (CNV) and the alterations in transcriptome or proteome. We found significant correlations between CNVs and expression levels of transcriptome or proteome.
Conclusions
Even with the relatively smaller sample size, the consistency between our analysis and that of TCGA supported the representativeness and reliability of our sequencing data. Our work shades light in understanding the regulatory network between genomics, transcriptomic and proteomics in Taiwanese oral cancer.

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Identification of urinary biomarkers for metastatic colorectal cancer

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Background
Colorectal cancer (CRC) metastasizes to the liver in approximately 50% of patients. Without treatment the median survival of these patients is less than eight months. Currently, there are no accurate predictive markers for metastatic CRC. The diagnosis of liver metastases mainly depends on imaging examination, therefore, it is costly and inconvenient for long-term monitoring. Urine is the only body fluid by virtue of noninvasive, easy collection at any time and large amounts of available volume. It can reflect the alternation of physiology and pathology in various organs of human body.
Methods
Here, a comprehensive urinary proteomic analysis of four groups of individuals (healthy control, CRC without metastasis, CRC with lymph nodes metastasis and CRC with liver metastasis) is performed using the tandem mass tag (TMT)-10 labeled 2D-LC-MS/MS strategy. Subsequently, potential urinary biomarkers were selected to further independent validation and functional experiments.
Results
In the discovery phase, a total of 1845, 2057, 2574 and 2250 proteins were identified in the healthy control, CRC without metastasis, CRC with lymph metastasis and CRC with liver metastasis groups, respectively. There were 998 proteins observed in all 4 groups. After comparison with healthy controls, the IPA analyses showed that actin cytoskeleton and integrin signaling pathways were markedly enriched in the disease groups. Meanwhile, the CRC groups showed decreased apoptosis and necrosis and enhanced proliferation. The independent validation showed that one of the candidate biomarker, HGS, had higher concentration in urine of patients with CRC liver metastasis. In addition, HGS knockdown decreased the migration ability while having no effect on cell proliferation in HCT116 cells.
Conclusions
Urine can be a good resource for the discovery of disease biomarkers. HGS may serve as a potential urinary prognostic biomarker for metastatic CRC, and contributes to cell migration.

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Role of posttranslational modifications on potential blood-based protein biomarkers in Alzheimer's disease

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Background
Posttranslational modifications (PTMs) are an integral part of a protein that regulates cellular behaviour. Despite recent studies, the role of PTMs in blood-based biomarkers in Alzheimer's disease (AD) pathogenesis is not well known. The main objective of this study is to identify specific PTMs on potential protein biomarkers in blood, to establish an accurate and reliable early diagnostic test for AD.
Methods
Human blood plasma samples from Australian Imaging, Biomarker and Lifestyle flagship study of aging (AIBL) were used in this project. These samples are categorised by their PiB-PET test values from high to low and in to AD, mild cognitive impairment (MCI) and healthy controls at the time for recruitment. Albumin depleted samples were fractionated using the OFFGEL 3100 fractionator on pH gradient (IPG) strips, separating in to 24 wells between pH 3-10. All fractions were trypsin digested, labelled with Tandem Mass Tags (TMT) and combined in to 10-plexs for analysis using Orbitrap LC-MS. Protein identification was done using 'PEAKS' bioinformatics software.
Results
A total of 54 proteins along with their PTMs were identified from two fractions using Uniprot database. These fractions were selected due to several proteins of interest as potential biomarkers. Proteins that reported highest peak intensities were Albumin, A2M, CO3, ITIH2, IGHA1, HPT, FIBG, CFAH, IGHM, HEMO, VTNC, C4BPA, FIBB, FIBA, CFAB, GELS, ITIH2, IGKC, ITHI1, IGHA1, THRB, and IGLL5.
Conclusions
This study shows the potential of combining OFFGEL, TMT and Orbitrap LC-MS for better understanding of PTMs compared to conventional 2D- gel electrophoresis. Thus, a robust research workflow was established to study a subset of AIBL cohort. Understanding unique signatures of PTMs among individual with low to high amyloid load warrants the identification of reliable blood-based biomarkers for AD.
Keywords
Alzheimer's disease, Posttranslational modifications, Tandem Mass Tag (TMT), OFFGEL fractionation, Orbitrap mass spectrometry

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Quantitative proteomics reveals temporal changes of signal pathways in BV2 microglial cell activation

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Background
The development of systematic proteome quantification techniques in systems biology research enables in-depth analysis of cellular systems. Therefore, we have designed an approach that can continuously analyze using a single platform and applied it to the activated microglia cellular system. Microglia become activated when their homeostatic microenvironment is disrupted. While there are varying degrees of microglia activation, we are focusing on a pro-inflammatory reactive state induced by exposure to stimuli like LPS and IFN-γ.
Methods
In this study, dimethyl-labeled proteomics and label-free PRM were used to present a systematic approach to proteomics that allows simultaneous quantification and targeted quantification on a single platform.
Results
We identified a total of 5497 proteins in whole cell proteome and 4938 proteins in secreted proteins associated with activated BV-2 mouse microglia cell line with LPS or IFN-γ using improved shotgun proteomic approach. From the differentially expressed proteins in stimulated microglia, we were able to classify pathways related to immune-inflammatory response and metabolism. The use of label-free PRM approach made it possible to comprehensively measure the hyper-multiplex quantitative value of each protein with high resolution mass spectrometry. Over 450 peptides corresponding to pathway proteins and directly or indirectly interacted proteins via the STRING database were able to quantify using the label-free PRM in a single run. Moreover, we performed longitudinal quantification of secreted proteins during the detrimental activation of microglia which releases neurotoxic molecules mediated neuronal cell loss in the brain region.
Conclusions
The data from this experiment suggests that latent biomarkers associated with neurodegenerative diseases can be discovered by presenting and analyzing a pathway network model of proteins. Furthermore, this systematical quantification platform has great potential for large-scale targeted analysis.
Keywords
Systems biology, Dimethyl labeling quantification, Label-free PRM, Microglia activation, Network analysis.

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Quantitative dot blot analysis (QDB) reveals the variation of housekeeping proteins at population level

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Introduction and Objectives
In proteomics research, housekeeping proteins are considered constantly expressed in cells and tissues. However, recent studies indicate that the expression level of housekeeping proteins presents significant variation. In this study, we intend to further investigate the variation of housekeeping proteins at population level with recently developed Quantitative dot blot analysis (QDB) with putative solution.
Methods
The entire prostate glands were isolated from 39 20-weeks prostate cancer (TRAMP) mice and their litter mates. The content of multiple proteins CAPG, tublin, B-actin, Lamin B, and GAPDH from the tissue lysates were investigated with Western blot analysis and QDB method. The absolute content of CAPG and Tublin was also determined using QDB method and BCA protein determination kit.
Results and Discussion
The expression levels of tubulin and other housekeeping proteins were not consistent with the total protein amount of lysate, as determined by either the BCA or Bradford assay. Using QDB analysis, we were able to show as much as 49 fold differences in tubulin level among these samples. The expression profile of CAPG level, either expressed as the ratio between CAPG and Tubulin, or over total protein amount determined by BCA assay, was significantly different.
Conclusion
The housekeeping proteins can present significant variation between individual subjects. Conclusions based on relative measurements are inherently limited. Measurement of absolute contents of individual proteins are necessary to present an unbiased picture at proteomics level.

Keywords: quantitative proteomics, BCA, House keeping gene, QDB.

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Monitoring protein refolding process by disulfide linkage analysis of the intermediates using mass spectrometry

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Disulfide bond is one of major post translation modifications for protein skeleton construction and folding maintainess. Recently, using solid phase peptide synthesis (SPPS) to generate peptide drug has been applied in pharmaceutical industry. For cysteine-rich peptides, analysis of correct disulfide bonds can be challenging. In this study, a well-developed platform using mass spectrometry was applied to profile the disulfide linkages of a synthetic peptide toxin, cardiotoxin A3 (composed by 60 amino acids with 4 disulfide bonds), during the refolding process. The bio-active cardiotoxin A3 product with correct folding was obtained by using an optimized dilution refolding method with the presence of GSSG/GSH in weak alkaline condition (phosphate buffer, pH 7.5, containing 1:3 mM ratio of GSSG/GSH). Based on the MS data, we were able to determine the protein fraction that generates exact disulfide linkage patterns as those found in native CTXA3, and the manufacturing conditions for peptide production were also optimized. The supplementary assays proved that the synthetic peptide product exhibited similar biochemical property and cell penetration activity. The results indicate that bioactive peptides with correct disulfide linkages could be obtained from SPPS approach with appropriate folding condition. It will be beneficial for the manufacturing of cysteine-rich biologics in biopharmaceutical industry. Eventually, this MS-based disulfide analysis platform provides significant information for protein structure-function study, and can serve as examination tool for cystein-rich bioproduct qualification.

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Identification of Serum Autoantibodies for Esophageal Squamous Cell Carcinoma Using Human Proteome Microarrays

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Background：

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant gastrointestinal cancer. Due to the asymptomatic nature of early stage ESCC and lack of effective screening strategies, most patients present with overt advanced disease. Currently, there are no established serological tumor markers for ESCC in clinic. Autoantibodies are considered as a kind of new serological markers for cancer diagnosis. Compared with the corresponding tumor-associated autoantigens (TAAs), antibodies are likely to be more stable with a longer half-lives in blood. Moreover, they show increased levels in very early cancer stages. To overview the serum autoantibodies in ESCC and healthy controls, to find new autoantibody biomarkers for the early detection of esophageal cancer.

Methods：

Here, the Human Proteome Microarrays which contain 19,394 recombinant proteins were used to comprehensively discover the differentially expressed serum autoantibodies between ESCC and cancer-free controls. Subsequently, some new autoantibody biomarkers for diagnosis ESCC were independently validated using western blot. Furthermore, the functional roles of BACH1 in ESCC were investigated.

Results：

A total of 201 autoantibodies relate to the early detection of ESCC and 215 kinds of autoantibodies relate to lymph node metastasis were identified using Human Proteome Microarrays. Three serum autoantibodies consisting of BACH1, PNMA2 and RAD23B provides high diagnostic performance for differentiate ESCC patients from healthy individuals ($P<0.05$). The preliminary functional studies showed that decreased BACH1 inhibit migration of ESCC cells by downregulating metastasis-related genes CXCR4 and MMP1.

Conclusion：

Three serum autoantibodies against BACH1, PNMA2 and RAD23B may serve as potential biomarkers for early detection of ESCC. BACH1 may function as an oncogenic driver and a potential therapeutic target in ESCC.

Keywords: Human proteome microarray; Esophageal squamous cell carcinoma; Early diagnosis; Autoantibodies; Tumor marker

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Stroma Liquid Biopsy - The Next Wave of Proteomic Profiles for Cancer Biomarkers

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Over the past few years, the concept of liquid biopsy has generated much scientific and commercial enthusiasm. This is because a liquid biopsy starts with a very accessible sample type: a body fluid, typically blood, rather than a surgically extracted tissue. Once available, a liquid biopsy can then be analyzed in a variety of ways to provide for example, a landscape of cancer-associated DNA mutations. Yet, the current liquid biopsy menu evaluates only genomic data which comes from a largely reductionist view that tumors form and progress only through the collection of its immortalized cells. These contributions notwithstanding, it is now overwhelmingly apparent that throughout cancer progression, there are necessary adaptive microenvironments to support metastatic disease. We now present evidence that some of the essential interactions between stroma and proliferating cells can, in part, be monitored through the protein response that tracks into the vascularized tumor and re-proportions the extracellular proteins (serum) found in the general blood circulation. This rewiring of the blood circuitry is measurable even at very early stages of cancer, for many if not most primary tumors. In the five primary tumors characterized to date, these biomarkers produce a pattern of dysregulation within three interconnected pathways involving coagulation, complement cascade and acute-phase inflammation. Using targeted LC-MS proteomic analyses, we advance that a “stroma liquid biopsy” will be orthogonal to and complementary with more conventional liquid biopsies based on nextgen sequencing and circulating DNA. This new observational window will help generate a more comprehensive profile of progressive disease, providing opportunities in monitoring risk factors, early detection, prognosis, recurrence, and guidance for therapeutic decisions.

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Potential biomarkers in the urine of babies with ROP

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Background: Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina predominantly affecting the premature, low birth weight infants and is one of the leading causes of preventable blindness worldwide. Here, we studied the proteomic differences in ROP urine using High Resolution Mass Spectrometry-based quantitative proteomics.

Methods:

1. Sample Collection – Urine samples were collected from preterm babies using minicoms. The collected samples were stored at -80 deg C. Urine samples of four babies with Retinopathy of prematurity were compared with urine samples of 3 preterm babies who did not develop ROP.

2. Proteomic analysis – Equal amounts of protein from control samples and ROP samples was subjected to trypsin digestion and labelled individually with TMT tags. The labelled samples were pooled and subjected to LC-MS/MS/MS analysis in triplicates. Each ROP urine sample was compared separately with each control (R4 x C3). Search algorithms such as Mascot and Sequest were used to search the MS data against human protein database. Peptide identification was at MS2 level and quantification at MS3 level. A fold change of ≥2 fold was considered as differential expression.

3. Statistical analysis – Statistical significance for each ROP and control pair was determined using the Holm-Sidak method with alpha = 5.0%.

Results: A total of 1336 proteins were identified. There was a more than 2 fold increase in the abundance of 8 proteins across all 12 comparisons. A total of 20 molecules were found to be statistically significant ($p<0.05$) across the 12 pairs of comparisons.

Conclusions: 20 proteins were significantly differentially expressed and 8 of these 20 were significantly elevated. These differentially expressed proteins could serve as potential biomarkers for ROP.

Key words: prematurity, urine proteomics,

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Omics Discovery Index: Supporting multi-omics analysis with public data

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Background: Currently, omics data are being produced at an unprecedented rate. This presents new challenges, including finding ways for linking and providing access to them. Specifically, finding datasets from different omics approaches that can complement a given proteomics study can be challenging. In our view, a dedicated resource enabling the aggregation of omics datasets, an analogous resource to PubMed for scientific publications, is needed.

The Omics Discovery Index (OmicsDI - http://www.omicsdi.org) is an open-source platform that can be used to discover and access omics datasets.

Methods: A set of algorithms and pipelines were developed to annotate and connect datasets from different public repositories and omics approaches. An exchange infrastructure including standard file formats, APIs and a web application, facilitates data retrieval.

Results and Discussion: By May 2017, eleven different repositories from four different omics approaches had been integrated into OmicsDI, including all ProteomeXchange ones (transcriptomics: 67,361; genomics: 8,093; proteomics: 6,281; metabolomics: 847).

Using their metadata, OmicsDI connects experiments that are deposited in different repositories that are part of the same multi-omics experiment. By May 2017, 4,476 datasets had been labelled as “multi-omics” (e.g. including more than 60 proteomics datasets in PRIDE). Also, metadata are used to connect datasets using disease, cell type or tissue annotations. Furthermore, the OmicsDI similarity algorithm can link similar datasets by comparing the list of identified proteins that are shared among them (e.g. see PRIDE dataset PRD000269, http://www.omicsdi.org/dataset/pride/PRD000269).

Conclusion: By integrating omics datasets in one central infrastructure, OmicsDI has become a unique resource enabling the finding, linking and more efficient reuse of public omics data. Also, it introduces modern features such as access metrics and discovery of related datasets, an analogous functionality to what we now take for granted in PubMed and Europe PMC for scientific publications.

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Protein crossroads: Dementia vs. Alzheimer's Disease

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Background. Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common cause of dementia. AD is a pressing public health problem with yet, no effective treatment. As aging is the most significant risk factor for AD, we hypothesis that aging-induced memory loss and additional deterioration pathways is due to citrullination which is enhanced with development of AD disease. Identification of these citrullinated driven pathways as potential mediators of brain dysfunction in AD may lead to the development of effective treatments for this disease.
Methods. The 5XFAD mouse model, with five early-onset familial AD (FAD) mutations, display substantial amyloid plaques and neurodegeneration was compared to their non-transgenic (Ntg) littermates. The aging- and AD-related memory impairments were assessed based on the contextual fear test (5XFAD intact memory status, n=10, 5XFAD impaired memory status, n=10, Ntg intact memory status, n=10, Ntg impaired memory status, n=10). The TripleTOF 5600 mass spectrometry using DIA acquisition and combined with integrated citrullinated pipeline was used to quantify total proteins and citrullinated proteins across all groups.
Results. 234 and 283 proteins were altered due to the memory status (intact vs. impaired) in Ntg and AD ($p < 0.05$), respectively. Of these proteins, 23 were increased and 24 were decreased with impaired AD compared to Ntg subjects. The proteins with altered levels in the AD brain vs. Ntg represent a variety of pathways suggested to be involved in the disease pathogenesis, including proline degradation, NADH repair, signal transduction and arginine degradation. Citrullinated proteins were presented at brains with intact memory indicate a potential physiological role but which were also altered in animals with advance memory loss. Pathways analysis highlighted neuron degradation and impaired mitochondrial biogenesis.
Conclusions. Overall, we provide a list of citrullinated protein and biological pathways that may underlie mechanisms for aging- and AD-related memory impairments.

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Differential Protein Expression Related to Testosterone Deficiency in Healthy Men. A Quantitative Proteomics Study.

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Background
Testosterone deficiency, also known as hypogonadism, afflicts approximately 30% of men aged 40-79 years and is associated with decreased fertility and, usually, displays unspecific clinical symptoms. Hypogonadism is also associated to major diseases such as cardiovascular disease and diabetes type 2. The current test for quantifying testosterone is an antibody binding technique. However, this measurement does not accurately correlate to the activity of the hormone. So far, there are no available biomarkers of the activity of testosterone, which greatly limits diagnostics and treatment outcome.
Methods
In our initial proteomics study, we analyse plasma samples (the top 7 proteins depleted) from healthy volunteers (men 20-30yrs, n=30) with pharmacologically induced testosterone deficiency in order to identify new potential biomarkers of testosterone activity. We analyse three conditions (“normal” testosterone, “zero” testosterone and “high” testosterone) with ten pooled samples (each pool n=3).
Results
A total of 77 out of 652 proteins were statistically differentially expressed between conditions, suggesting that we have identified testosterone sensitive proteins that we will utilize in future studies in order to develop a multiple reaction monitoring (MRM) assay.
Conclusions
Testosterone changes cause clear differences at protein levels.
There is good correlation between differentially expressed proteins and clinical data from volunteers.
Most of differentially expressed proteins have broad tissue profiles.
Keywords
Testosterone, proteomic.

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Whole Proteome Sequence Map of Early Stage Human Ovarian Follicular Fluid

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Background

Small antral follicles are considered the first sign of a new stage of maturation of follicles. The antrum of the follicles is surrounded by follicle fluid, which volume reflects the stages of oocyte development and the degree of follicle maturation.

Methods

Orbitrap Q Exactive Mass Spectrometer, Top 7 abundant protein depletion, Multidimensional chromatographic.

Results

Previously, only follicular fluid from follicles larger than 10 mm in diameter has been analyzed resulting in a high content of plasma proteins. Consequently, this results in an extremely high dynamic range with protein identifications in the hundreds range. Here, we present the first proteomics analysis of small follicles (3-8mm) with 1750 proteins groups identified

Conclusions

We have now identified the highest to date number of proteins in follicular fluid. Our study report the first proteins characterization by mass spectrometry of small antral follicles. The data contains a lower percentage of extracellular proteins compared to previous reports. The data provide a high quality dataset with some poorly characterized proteins, which is useful like spectral library for future experiments of quantitative proteomics.

Keywords

Antral Follicle, Proteomics, follicular fluid

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PECAsuite: a comprehensive software package for time-specific, multi-layered analysis of gene expression regulation

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Background
Profiling of simultaneous time series measurements of mRNA and protein concentrations is becoming a common practice. However, there is a need for algorithms to handle measurement noise, time point dependency, and different regulatory layers. We previously developed a method called Protein Expression Control Analysis (PECA), which uses mass action models to track changes in the ratio of synthesis and degradation rates and calculates the probability of regulation change points. Here, we present extensions of the PECA framework that enable streamlined analysis of various datasets.

Methods
The new features in PECA encompass data pre-processing, modeling, and post-processing. This includes time series data smoothing and missing data imputation, and time-dependent gene set enrichment analysis. More importantly, we built three new modules for modeling: (i) PECA-N to incorporate biological networks in calculation of regulation changes; (ii) PECA-PS model to estimate synthesis and degradation rates using pulsed SILAC data; and (iii) PECA-R model to estimate rate parameters from label-free proteomics data and transcriptomics data.

Results
The PECA software suite enables flexible and sensitive analysis of various datasets. Using a label-free dataset for protein folding stress (Cheng, MSB 2016), we show that PECA-N model incorporates biological network information to jointly boost significance scores for densely interconnected genes, improving overall sensitivity in identification of significant regulatory events. In a pulsed-SILAC dataset for LPS stimulation (Jovanovic, Science 2015), PECA-PS model not only estimates separate rates for synthesis and degradation using pulsed SILAC data as well as differential equations-based methods, but also calculates significance scores at specific time points. Finally, we compare the PECA-PS output to the rates estimated by PECA-R model from label-free proteomics data and show that protein synthesis and degradation rates can be inferred from label-free data, without the need to perform costly pulsed labeling experiments.

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Proteomics of Urine AQP2-bearing Exosome

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Background:
Aquaporin 2 (aquaporin 2: AQP 2) is a significant water channel, which plays an important role in water reabsorption of the kidney. AQP2 is exclusively expressed in the collecting duct principal cells and the protein translocation between intracellular vesicles and plasma membranes is mediated by antidiuretic hormone vasopressin stimulation. Since the molecular mechanism still remains unclear, AQP2-bearing urine exosomes were analyzed by proteomics to identify proteins associated with AQP2 to elucidate the mechanism since recent reports showed AQP2 proteins in urine exosomes.
Methods:
Human urine exosomes were isolated by the ultracentrifugation method. After freezing and thawing of the exosomes, AQP2-bearing membranes were separated by co-immunoprecipitation with AQP2 antibody. Both the AQP2-bearing exosome membranes and those not separated by AQP2 antibody were solubilized in urea, digested with trypsin and analyzed by LC-MS (Orbitrap Fusion). Proteins were identified by Mascot search engine (FDR <0.01). The identified proteins were confirmed to be localized in the kidney by using the Human Protein Atlas database and were analyzed by pathway analysis tools (DAVID and IPA).
Results:
By the LC-MS, more than 1,500 proteins were identified in the control urine exosome membranes and 150 proteins in the AQP2-bearing exosome membranes. By comparing these proteomics data, 13 proteins were predicted as characteristic proteins associated with AQP2. The Human Protein Atlas immunohistochemistry images showed that most of these proteins were localized in the collecting duct in the kidney. Pathway analysis elucidated that the AQP2-bearing exosome membrane proteins were significantly involved in the endocytosis pathway.
Conclusions:
AQP2-bearing urine exosome membranes were selected by co-immnoprecipitation using AQP2 antibody and analyzed by LC-MS. The proteins, uniquely identified in the AQP2-bearing membranes, are presumed to associate with AQP2 molecules and involved in the AQP2 protein translocation and regulation of water reabsorption.
Keywords:
Urine, Exosome, AQP2

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Analyses of glycans on haptoglobin in sera of patients with various types of cancer

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Background:
Most serum proteins are glycosylated. Especially, fucosylation is an important event because it results in the formation of blood-type antigens and cancer-associated carbohydrate antigens. Previously, we reported that fucosylated N-glycans on haptoglobin (Hpt) in the sera of patients with pancreatic cancer were increased. After this, an increase in fucosylated Hpt has been reported in various types of cancer. To ascertain if characteristic fucosylation is observed in each cancer type, we undertook analyses of N-glycans on Hpt in the sera of patients with various types of cancer.
Methods:
Hpt was purified from sera of patients with five types of gastroenterological cancer (esophageal, gastric, colon, biliary, pancreatic), a non-gastroenterological cancer (prostate) and normal controls using anti-Hpt antibody. Hpt has four glycan-binding sites (Asn184, Asn207, Asn211, Asn241). The site-specific analysis of N-glycans and the linkage analysis of fucosylation were performed by LC(ODS)-ESI MS and LC(Graphitized Carbon)-ESI MS, respectively.
Results:
Site-specific analysis using glycopeptide samples showed monofucosylated N-glycans were significantly increased at all glycosylation sites in all cancer samples. Moreover, difucosylated N-glycans were detected at Asn 184, Asn207 and Asn241 only in cancer samples. Remarkable differences in N-glycan structure among cancer types were not observed. Linkage analysis using alditol samples released from Hpt showed Lewis-type fucosylated N-glycan was increased in gastroenterological cancer samples, but core-type fucosylated N-glycan was increased in prostate cancer samples. In metastatic prostate cancer, Lewis-type fucosylated N-glycan was also increased. In biliary tract cancer samples, only Lewis-type fucosylated N-glycan was increased in gallbladder cancer samples, but core-type fucosylated N-glycan was also increased in bile duct cancer samples.
Conclusions:
These data suggest that the original tissue/cell producing fucosylated haptoglobin is different in each cancer type and linkage of fucosylation might be a clue of primary lesion, thereby enabling a differential diagnosis between gastroenterological cancers and non-gastroenterological cancers.
Keywords:
haptoglobin, fucosylation, cancer

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PepQuery: a peptide-centric search engine that makes proteomics data directly usable to the genomics community

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Background
Cancer genomics studies have identified a large number of genomic alterations that may lead to novel, cancer-specific protein sequences. Proteins resulted from these genomic alterations are attractive candidates for cancer biomarkers and therapeutic targets. The leading approach to proteomic validation of genomic alterations is to analyze tandem mass spectrometry (MS/MS) data using customized proteomics databases created from genomics data. Such analysis is time-consuming and requires thorough training and detailed knowledge in proteomics data analysis, leading to a gap between MS/MS data and the cancer genomics community. Here, we present a new search engine PepQuery, which does not require customized databases and allows quick and easy proteomic validation of genomic alterations.

Methods
In PepQuery, each input peptide is searched against an MS/MS dataset specified by a user and candidate peptide-spectrum matches (PSMs) are identified. Next, the candidate spectra are searched against a reference protein database, and those with a better match to sequences in the reference database than to the target sequence are removed. Remaining PSMs are subjected to further statistical evaluations.

Results
We used three public datasets to evaluate the performance of PepQuery for both novel sequence and mutation validation. The average false positive rate was 0.75% and the average true positive rate was 96.4%. We implemented PepQuery as both standalone and web-based tools. The web version provides direct access to all proteomic data sets generated by the Clinical Proteomic Tumor Analysis Consortium (CPTAC). Searching one peptide against about 10 million MS/MS spectra took less than 15 seconds with 16 threads.

Conclusions
We have developed PepQuery, a peptide-centric search engine for novel peptide identification and validation. We demonstrated the sensitivity and specificity of PepQuery using public datasets. We anticipate that PepQuery will significantly increase the usage of MS proteomics data in the genomics community.

Keywords
Proteogenomics, Search engine, Cancer

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Role of α2,6-Sialylation in inhibiting adipogenesis

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Our group has been interested in the role of N-glycan branching in relation to various diseases such as cancer, Alzheimer's disease, and COPD. It is well known that adipose tissue plays critical roles in obesity and related diseases such as diabetes and cardiovascular diseases. Previous reports suggest that glycans are involved in obesity-related diseases, but what type of glycan regulates adipogenesis during obesity remains still unknown. Here we examined quantified mRNA levels of 167 genes (encoding 144 glycosyltransferases and 23 related enzymes) in visceral adipose tissues (VATs) from control mice and high fat diet (HFD)-induced obese mice. We found that a gene encoding β-galactoside α2,6-sialyltransferase-1 (St6gal1), a key enzyme responsible for the biosynthesis of α2,6-linked sialic acid in N-linked glycans, was most downregulated in VATs from obese mice. We confirmed the reduction in α2,6-sialic acid in VATs from obese mice and differentiated adipocyte model 3T3-L1 cells. Using LC/MS analysis, integrin-β1 was identified as one of the target α2, 6-sialylated proteins in adipose tissues. Phosphorylation of its downstream molecule focal adhesion kinase (FAK) was found to be decreased after HFD feeding. St6gal1 overexpression in differentiating 3T3-L1 cells inhibited adipogenesis with increased phosphorylation of FAK. Furthermore, St6gal1 knockout mice exhibited increased bodyweight and VAT weight after HFD feeding. Treatment with a DNA methyltransferase inhibitor downregulated St6gal1 during adipogenesis indicating an involvement of epigenetic DNA methylation in St6gal1 silencing. These results suggest that ST6GAL1 has an inhibitory role in adipogenesis through integrin-β1 activation and this may open a new insights into the roles and regulation mechanisms of glycans in adipocytes during obesity.

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Label-free quantitative proteomic analysis to investigate the effect of heat stress on Panax ginseng leaves

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Ginseng is one of the well-known medicinal plants, exhibiting diverse medicinal effects. Its roots possess anticancer and anti-aging properties and are being used in the medical systems of East Asian countries. Ginseng is grown in low-light and low-temperature conditions and its growth is strongly inhibited at temperatures above 25 °C. However, the molecular responses of ginseng to heat stress are currently poorly understood, especially at protein level. Therefore, here we utilized a shotgun proteomics approach to investigate the effect of heat stress on ginseng leaves. Total proteins were isolated from control (25 °C) and ginseng plants exposed to 35 °C for 1 and 3 days and subjected to in-solution trypsin digestion. A total of 3359 ginseng proteins were identified when searched in an in-house developed RNA-seq (PAC-BIO) database. Obtained data were filtered with ≥2 unique peptides and identifications less than 50% missing values were discarded, which narrowed down the identification list to 1143. Label-free quantitative proteomic analysis using MaxQuant led to the identification of 712 differentially modulated proteins (multiple ANOVA test, Benjamini–Hochberg controlled FDR<0.01) of which 284 proteins were higher than threshold (>1.3 and <0.7 for increased and decreased modulation respectively). Functional annotation of the proteins with increased abundance showed these were mainly associated with the antioxidant and translation regulator activities while proteins related to receptor activity and structural binding activity showed decreased abundance. PANTHER pathway analysis showed proteins related to the ATP synthesis, glycolysis, and ubiquitin-proteasome pathways were downregulated while purine biosynthesis, leucine biosynthesis, pyruvate metabolism and TCA cycle associated proteins were increased in abundance. Several other proteins including calcium binding proteins, chaperones, cytoskeletal proteins, signaling proteins, transcription factors and transfer/carrier proteins were specifically down-regulated. These results increase our understanding of heat stress responses in ginseng leaves at the protein level, providing a resource for the scientific community.

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Glycosylation changes of cell membrane proteins in pancreatic cancer

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Background:
Glycosylation is a critical post-translational modification of proteins and contributes to structural stabilization and various functionalization of proteins. Glycan structures change by diseases such as inflammation and tumor. Therefore, structural changed glycan on proteins could be useful targets for diagnosis and treatment. In this study, we comprehensively analyzed glycan structures on pancreatic cancer tissues to investigate cytoscreening marker and therapeutic target for pancreatic cancer.
Methods:
A piece of pancreatic cancer tissue removed at the time of the surgical procedure was used in this study. Tumor section and non-tumor section were separately homogenized. The homogenate was subjected to ultracentrifugation and detergent-phase partitioning to obtain cell membrane proteins, and the proteins were dotted on PVDF membrane. N-Glycans were released by PNGaseF, and were labeled with aminoxyTMT. Subsequently O-glycans were released by β-elimination as alditols. Both of glycans were analyzed by LC-ESI MS.
Results:
N-glycans containing ABO antigens (H (O), -Galα1-2Fuc; A, H+α1-3GalNAc; B, H+α1-3Gal) were observed as major components in non-tumor sections, whereas they were barely observed in tumor sections. This change between in non-tumor and tumor sections was more highlighted in O-glycans. Moreover, O-glycans including core 2 type structure (-GalNAcα1-3Gal (α1-6GlcNAc)) were increased in tumor sections.
Conclusions:
It is already reported that activities of various fucosyltransferases change in tumor cells. We suggest that the reduced activity of fucosyltransferase 2 (FUT2, α1-2, involving synthesis of H antigen) results in decrease of ABO antigens in tumor section. In O-glycans, GlcNAc is transferred to -GalNAcα1-3Gal backbone which was not fucosylated by FUT 2 to form the core 2 type structure, and then subsequently undergoes modification of galactose and sialic acid to be maturated core 2 type structures. We suggest that is the reason why core 2 type structures of O-glycans increased in tumor sections.
Keywords:
glycan, ABO antigens, pancreatic cancer, biopsy tissue

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Optimization of phase transfer surfactant-aided in-solution digestion method for an automated large-scale sample preparation system

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Large amount of proteins (e.g., milligram scale) is often required for deep proteomic analysis that targets low-abundant molecules such as peptides with posttranscriptional modification (PTM). Usually, such a large-scale analysis requires laborious work and long time for sample preparation. It is expected that the method optimization based 96-well plate format can improve throughput of PTM-proteomics.
Compared with traditional in-solution digestion protocol using urea, phase transfer surfactant-aided in-solution digestion (PTS) protocol increases the solubility of hydrophobic proteins, enhances the activity of trypsin, and improves the membrane protein recovery rate and cleavage efficiency. However, two problems make it difficult to adapt PTS method to a 96-well plate format. One is that the volume of a trypsin reaction mixture can be too large to use the 96-well deep plate. Another problem is that vigorous mixing with organic liquid (i.e., ethyl acetate), which is performed in liquid-liquid extraction step for removing the surfactant, is not easy to do in a 96-well plate.
In this study, we employed the volume reduction method of the trypsin digestion step. Furthermore, we evaluated another method of the step of removing the surfactant based on acid-induced precipitation which can avoid vigorous mixing.
As a result, we found that the volume reduction by 1/5 in the trypsin digestion step did not affect the identification and quantification of peptides in Hela cell lysate. We also found that the acid-induced precipitation method in the step of the surfactant removal did not affect. Therefore we can overcome the two issues by our modified PTS method on the volume in the trypsin digestion step and on the step of the surfactant removal. The method enables the large-scale trypsin digestion in the 96-well format, suggesting that it can facilitate deep and high-throughput analysis of PTM-proteome.

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Highly sensitive detection of adaptation in Staphylococcus aureus applying chemical tagging of newly synthesized proteins

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Proteomic monitoring of stress adaptation reactions in S. aureus and other bacteria by proteomics has so far most successfully been done by 2-DE employing 35S-methionine pulse-labeling because of its ability of recording changes in protein synthesis. Such protein synthesis measurements have not been adopted for mass spectrometry focused approaches for S. aureus. During infection S. aureus is confronted with the innate immune system, where oxidative stress is a major threat for the bug. Understanding the molecular mechanisms of ROS adaptation of the pathogen is important since it will help to determine new anti-staphylococcal strategies in the future.

The stress adaptation to 2mM diamide or 500µM hypochlorite was investigated in methionine-free medium via the QuaNCAT approach by applying click-iT chemistry with L-azidohomoalanine in combination with SILAC. The samples were either directly digested or enriched using the click-iT chemistry and subsequently digested, analysed by LC-MS and compared with each other or to previously published data.

The comparison of the QuaNCAT results versus previously published data gathered by 2-DE on diamide stress response in S. aureus revealed increased synthesis of 86 proteins, which have not been detected before and some of which are important stress adaptation proteins (e.g. SodA, AhpF). The response to diamide or hypochlorite stress revealed 96 or 81 proteins up-regulated after stress, which could not be detected by a conventional LC-MS approach alone. Among them are important oxidative stress proteins (e.g. KatA, AhpC, MsrA) or enzymes involved in the protein quality control and its regulation (e.g. ClpB, ClpP, McsB, CtsR).

The established QuaNCAT workflow for S. aureus revealed new insights in the oxidative stress response of the pathogen and is superior to a LC-MS based relative quantification without the enrichment of synthesized proteins for the detection of stress adaption processes. The workflow enables sensitive recording of adaption to environmental cues.

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Comparative study on skeletal muscle proteins from various vertebrate and invertebrate animals by agarose 2-DE

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Skeletal muscles of vertebrates are mainly composed of fast-twitch and slow-twitch fibers that differ in their contractile properties. The two muscles expressed different isoforms of contractile proteins including myosin heavy chain and light chain, and regulatory proteins, such as troponin and tropomyosin. The first-twitch fibers express higher levels of cytoplasmic glycolytic enzymes to allow fast glycolysis in anaerobic respiration. In contrast, the slow-twitch fibers have a highly density of mitochondria expressing mitochondrial ATP synthase within the cells. These differences of muscle proteins are easily detected by two-dimensional gel patterns. Some invertebrate muscles, such as crab and lobster claws, have fast-twitch and slow-switch fibers that differ in their myosin and troponin isoforms. In this work, we compared between fast-twitch and slow-twitch muscle proteins from various vertebrate and invertebrate animals by an agarose two-dimensional gel electrophoresis (agarose 2-DE), that is sufficiently good at separating high-molecular-mass proteins with molecular masses as large as 600kDa. In crab claws, the slow-switch muscle has a highly content of paramyosin, an invertebrate specific protein that is homologous to the rod portion of myosin heavy chain, and is localized in thick filament cores. However, the contents of the cytoplasmic glycolytic enzymes in the slow-twitch muscle were almost same as those in the fast-twitch muscle. These results suggest that the fast and slow muscles in invertebrate animals evolve independently with those in vertebrate animals.

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Digging more missing proteins using a depletion approach with ProteoMiner

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Background
Human Proteome Project (HPP) aims at mapping the entire human proteins with a systematic effort upon all the emerging techniques, which would enhance understanding of human biology and lay a foundation for development of medical applications. Till now, 2,579 missing proteins are still undetected even using the most sensitive approach of protein detection. Herein, we have proposed that depletion of higher abundance proteins could improve detection of lower abundance or smaller proteins in protein extracts, while ProteoMiner™ is an efficient kit for such depletion even though it was designed just for depletion of higher abundance proteins in serum.
Methods
NH_4HCO_3 (50mM) or Triton X-100 (1%) was used to extract proteins from human tissues or cell lines. The extracted proteins were captured by ProteoMiner beads and the proteins beyond the binding limits were expelled. The bound proteins on the beads were eluted and digested with trypsin. The tryptic peptides were fractionated and delivered to Q Exactive™ HF mass spectrometer for protein identification.
Results
Six mg of liver proteins treated with 50µL ProteoMiner beads resulted in 2.5% and 3.6% protein recovery for the NH_4HCO_3 and Triton X-100 extractions, respectively. After 20 fractions, approximately 6,000 proteins were identified, including 2 or 5 missing proteins in the two extraction with at least 2 unique peptides (≥9 a.a.) and protein FDR <1%. In these proteins, one protein was co-identified in the two extractions. The five proteins are assigned to membrane or secretary proteins and the other one is not confirmed by any experiment yet. Based on the preliminary results, more missing proteins are expected being identified once the proteins are extracted from different human tissues and cell lines with such depletion approach.
Conclusions
The ProteoMiner treatment to the extracted proteins is helpful to enrich and find missing proteins.
Keywords
ProteoMiner, Missing proteins, LC-MS/MS

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iOmicsPASS: Integrative-omics approach for Predictive Analysis with Subnetwork Signatures

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Background
mRNA expression data are often used as a surrogate measure of protein levels. However, the two are not always correlated and analysing each data-type yields different results. Hence, orthogonal information from each -omics data should be integrated. Availability of proteomic and transcriptomic data in large-scale clinical studies such as The Cancer Genome Atlas (TCGA) creates opportunities to better characterize the molecular landscape in complex human diseases. Also, past literature has accumulated a vast amount of experimental data validating molecular interactions and regulatory connections. This cumulative knowledge can be used to infer the biological relationship between different molecular levels in a large number of tumor specimens of cancer patients, while achieving the primary goal of phenotype prediction.

Methods
We developed a bioinformatics workflow, iOmicsPASS, which combines multiple –omics data to build predictive gene signatures in the space of biological networks for clinical outcome prediction. The predictive model leverages on the widely used “shrunken centroid” prediction method, where the patients' group centroids are searched with a network-based shrinkage method. For protein, mRNA, DNA copy number-based prediction, predictive multi-omics signatures of subtypes are constructed based on profiles consistent with the transcription factor regulatory networks for protein-mRNA targets and protein-protein interaction networks. Here we also proposed a novel method for pathway-level scoring of the subnetworks identified, to rank important and predictive biological pathways involved within each of the cancer subtypes.

Results
We illustrate iOmicsPASS using the invasive breast cancer cohort data in TCGA, with evaluation of predictive performance in an independent breast cancer cohort with microarray gene expression data only. Our results show the predictive subnetworks signatures identified across the subtypes by utilizing multi-omics data are not only biologically sensible, but also enables highly robust prediction of cancer subtypes, improving the current mRNA-based molecular subtyping.

Keywords
Predictive modeling, multi-omics data integration, pathway-level scoring

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Protein target identification of label-free bioactive small molecule

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Backgrounds
Identifying the protein target of bioactive small molecule is a pivotal step for developing therapeutic drug. However, conventional target identification methods need to modify the small molecule for immobilization. Accordingly, there have been a number of drawbacks in respect with alteration of activity, cost and time-wise. To overcome these limitations, target identification of label-free small molecule by combination of drug affinity responsive target stability (DARTS) and LC-MS/MS method is developed in this study.

Methods
SWATH analysis (Sequential window acquisition of all theoretical fragment ion spectra), Triple ToF (AbSciex, Triple ToF 6600+), Orbitrap (Thermo Fisher scientific, Orbitrap Elite), IP2 (Integrated proteomics pipeline) analysis, Western blotting (P62, LC3, and ß-actin), Lysotracker, Tandem LC3, SDS PAGE, CBB staining, Tyrosinase activity assay, viability assay.

Results
We have identified a natural small molecule (Compound A) that induce autophagy by increasing LC-3 conversion and P62 degradation with unknown target and mechanism. To address the underlying mechanism of the compound for its autophagy inducing activity, DARTS and LC-MS/MS analysis of in-gel digested proteome from w or w/o compound A treatment together with pronase was conducted. By comparing quantitated value for group of compound A, pronase, and pronase treated with compound A, both increased or decreased protein stability under pronase treated condition were identified. Among 26 candidates, tyrosinase binding protein (TBP) was reproducibly identified in the decreased protein stability by compound A. Notably, Compound A suppressed tyrosinase activity in a dose dependent manner. These results demonstrated that Compound A induces autophagy by binding to TBP and TBP could play a role in autophagy biogenesis.

Conclusions
In conclusion, this study suggests that DARTS and LC-MS/MS method could be an efficient way for identification of target protein of label-free small molecules.

Keywords: DARTS, LC-MS/MS, Autophagy, IP2, SWATH, P62, LC-3

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Oxidation of protein-bound methionine in Photofrin-photodynamic therapy-treated human tumor cells explored by quantitative proteomics approach

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In Photofrin-mediated photodynamic therapy (PDT), cell fate can be modulated by the subcellular location of Photofrin. PDT triggers oxidative damage to target cells, including the methionine (Met) oxidation of proteins. Here, we developed a new Met-containing peptide enrichment protocol combined with SILAC-based quantitative proteomics, and used this approach to explore the global Met oxidation changes of proteins in PDT-treated epidermoid carcinoma A431 cells preloaded with Photofrin at the plasma membrane, ER/Golgi, or ubiquitously. We identified 431 Met-peptides corresponding to 302 proteins that underwent severe oxidation upon PDT and observed overrepresentation of proteins related to the cell surface, plasma membrane, ER, Golgi, and endosome under all three conditions. The most frequently oxidized Met-peptide sequence was “QAMXXMM-E/G/M-S/G-A/G/F-XG”. We also identified several hundred potential Photofrin-binding proteins using affinity purification coupled with LC-MS/MS, and confirmed the bindings of EGFR and cathepsin D with Photofrin. The enzyme activities of both proteins were significantly reduced by Photofrin-PDT. Our results shed light on the global and site-specific changes in Met-peptide oxidation among cells undergoing Photofrin-PDT-mediated oxidative stress originating from distinct subcellular sites, and suggest numerous potential Photofrin-binding proteins. These findings provide new insight into the molecular targets through which Photofrin-PDT has diverse effects on target cells.

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Novel-platform for discovery of an angiogenesis inhibitor and identification of target on tissues using-MALDI-MSI analysis

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Background

The goal of our research is to identify new bioactive small molecules with cell-based screening and mode of action study using proteomics. For this aim, we identified and validated the protein target of small molecules to address the small molecule-protein target interaction in vivo level using MALDI-MSI (MALDI-mass spectrometry imaging) and IF (immunofluorescence) staining. This approach will provide new information on assessment of the pharmacokinetic and pharmacodynamic properties of bioactive small molecule and its target protein on tissues.

Methods

Cell-based screening assay with crude extracts library of natural plants, viability assay, tube formation assay, chemo-invasion assay, in vivo-CAM (chicken chorioallantoic membrane) assay, Western blotting (HIF-1α, phospho-RTKs, VR receptor, ERK, Akt), VEGF-ELISA, DARTS (Drug Affinity Responsive Target Stability), MALDI-MSI (LTQ Orbitrap XL, Thermo Scientific), IF (Immuno-Fluorescence) staining were conducted in this study.

Results

Previously, we identified a new natural small molecule, YCG185, as an angiogenesis inhibitor in vitro and in vivo without showing cell toxicity. Notably, YCG185 decreased the expression of HIF-1α and its target gene, VEGF. YCG185 specifically inhibited the phosphorylation-VR receptor and its downstream signaling, ERK and Akt and directly interacted with VR receptors in DARTS assay. The mode of actions of YCG185 was further investigated by analyzing the distributions of YCG185 and its target protein on xenograft tumor tissues using MALDI-MSI analysis and IF staining. As the results, MALDI-MSI drug image of YCG185 is highly co-localized with IF staining image of VR receptors on tissues, suggesting that YCG185 directly interacts with its target protein in vivo.

Conclusions

This novel platform of MALDI-MSI based drug imaging of a label-free anti-angiogenic small molecule and IF staining of its protein target could provide a novel insight into the interaction of drug and its target on tissues as well as the pharmacodynamic and pharmacokinetic properties of the compound in vivo.

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Deep phospho- and phosphotyrosine proteomics identified active kinases and phosphorylation network in Cetuximab-resistant colorectal cancer

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Abnormality in phosphorylation signaling is closely related to oncogenesis. Therefore, protein kinases, regulators of phosphorylated modification, has been studied as therapeutic targets. So far, many tyrosine kinase inhibitor (TKI) has been approved or is currently in the clinical trials, however, intrinsic and acquired resistance to TKIs increases burden on patients. Therefore, global phosphoproteomic analysis, especially phosphotyrosine (pY) proteome, could contribute to predict TKI sensitivity and overcome TKI-resistant cancer.
In this study, we performed deep phosphoproteomic analysis to elucidate the mechanism of colorectal cancer which has intrinsic resistance to cetuximab. In order to compare phosphoproteomic status between Cetuximab-sensitive cell lines (LIM1215 and DLD1) and Cetuximab-resistant cell lines (HCT116 and HT29), we obtained phosphoproteomic data with immobilized metal-ion affinity chromatography-based phosphoproteomic and highly sensitive pY proteomics. From the phosphoproteomic data, we found active kinase candidates in resistant cell lines with phosphorylation database, Uniprot, and kinase-substrate enrichment analysis. Then, we evaluated effect of active kinase candidates with siRNAs and specific inhibitors on cell proliferation.
Our phosphoproteomic methods identified 13411 phosphosites and 1308 pY sites from triplicate experiments. By using informatic analysis, 15 and 4 active kinase candidates were identified in Cetuximab-resistant HCT116 and HT29 cell, respectively. Especially, activation of SRC-PRKCD cascade were constitutively observed in HCT116 cell irrespective of Cetuximab treatment. Then, we validated the SRC activation in Cetuximab-resistant cell lines by suppressing SRC either with siRNAs or chemical inhibitors. Suppression of SRC significantly decreased proliferation of HCT116 and other Cetuximab-resistant cell lines. In summary, our phosphoproteomic approach could contribute to elucidation of the mechanism of drug resistance and discovery of novel target kinases for anti-cancer therapy.

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ASSESSING THE SELECTIVITY OF ANTIBODIES AGAINST VARIOUS AMYLOID-BETA SPECIES BY AN AUTOMATED CAPILLARY BASED IMMUNOASSAY

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Background: Various amyloid-beta peptides differing in their amino-termini have been described, which may be relevant for the pathogenesis of Alzheimer's disease and / or as potential novel biomarker candidates. We report here on the application of a Capillary Isoelectric Focusing Immunoassay (CIEF Immunoassay) to investigate the selectivity of antibodies raised against amyloid-beta peptides starting e.g. at Val(-3), Ala(2) or Phe(4).
Methods: The CIEF Immunoassay runs in an automated fashion on a "Peggy Sue" device from ProteinSimple. In brief, synthetic amyloid-beta peptides are separated by isoelectric focusing in microcapillaries according to charge, immobilized in a photochemical reaction and finally detected immunologically with primary antibodies against N-terminal epitopes in combination with secondary reagents ultimately generating a chemiluminescent signal. Additionally, urea SDS polyacrylamide gel electrophoresis followed by Western immunoblotting is applied.
Results: The CIEF immunoassay allows for very rapid and convenient antibody characterization in terms of selectivity for specific N-terminal variants of amyloid-beta. While some of the tested anti-amyloid-beta antibodies detected multiple peptide variants, others showed high selectivity for particular amino-terminal variants such as amyloid-beta (2-X), amyloid-beta (-3-X) or amyloid-beta (4-x).
Conclusions: The automated CIEF immunoassay represents a highly attractive tool for the characterization of antibodies against different N-terminal variants of amyloid-beta peptides.
Keywords: Alzheimer disease, Amyloid-beta peptide, Antibody characterization, CIEF Immunoassay

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Multiomics Data Integration using Ontologies

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Multiomics data integration is a concept seen frequently in bioinformatics. As is often the case with hot topics, the understanding of what the concept entails varies greatly between researchers and across disciplines. In an ideal world, multiomics data would perhaps be generated concomitantly, following a common experimental design. In reality, one research group may only have the technological expertise or infrastructure to either perform gene expression, proteomics or metabolomics measurements, and the data invariably becomes heterogeneous. However, much omics data is already in the public domain. Here I will discuss specifically how we use anatomical, chemical and bioinformatics ontologies to integrate previously published transcriptomics and proteomics data in model systems.

Anatomical ontologies exist for the common model systems, including C. elegans, Drosophila, zebrafish and mouse. Since in our research we mostly use zebrafish and mouse models, the focus will be on the ZFA/ZFS ontologies for zebrafish and MA for mouse. Some of the RNA-Seq transcriptomics and mass spectrometry based proteomics data was generated in our labs and those of our collaborators. We integrated this data with data in the public domain using the anatomically annotated gene observation data from the Zebrafish Information Network and several resources for gene expression in mouse. The taxonomies provided by the ontologies ensures semantic retention when traversing from one omics dataset to another. We also developed a tool, COMICS, to visualize and interact with anatomically resolved multiomics data in and across model systems.

In addition to the anatomical ontologies, we also used ontologies for chemical methods such as CHMO and bioinformatics ontologies like EDAM to model information and provide thesauri for text mining the scientific literature linked with public datasets. This completes the metadata picture and helps in automatically extracting the most relevant pieces of data for multiomics data comparisons.

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Glycosylation Analysis of Prostate Specific Antigen – Towards Improved Diagnosis of Prostate Cancer

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Background

Currently, early screening of prostate cancer (PCa) is based on detecting elevated plasma concentrations (> 3 ng/mL) of prostate specific antigen (PSA). Despite of its high sensitivity, the PSA test lacks specificity as other prostate related diseases can also cause elevated levels. Nonetheless, literature has described that PSA glycosylation could be a promising biomarker of PCa and therefore during this study a high-performance PSA glycosylation assay was established.

Methods

A commercially available PSA standard (1.5 µg) was reduced and alkylated prior to enzymatic digestion (trypsin). Analysis was performed by capillary electrophoresis electrospray ionization coupled to mass spectrometry (CESI-MS) and compared with a traditional MALDI-TOF-MS approach (PNGase F released N-glycans treated with ethyl esterification).

Results & Discussion

The analysis of tryptic PSA showed that separation was based on the glycan composition, especially the degree of sialylation. Furthermore, results showed isomer separation of the differentially linked sialic acid. This is phenomena is specifically interesting as sialic acids are often found at the terminus of the glycan chain, serving as a binding site for human lectins, toxins and pathogens in a linkage-specific manner. For example, alpha2,3-linked sialic acids seem to be a hallmark of malignant PCa. In order to structurally assign the isomers, the sample was treated with alpha2,3-sialidase for determining sialic acid linkages. Notably, CESI-MS identified a significantly higher number of glycoforms of PSA on a single N-glycosylation site (N69) compared to MALDI-TOF-MS analysis of released N-glycans [75 by CESI-MS (glycopeptides) compared to 37 by MALDI-TOF-MS (N-glycans)] highlighting the gain in analytical depth obtained with this approach.

Conclusions

We established a high-performance PSA glycosylation assay which features sialic acid isomer differentiation for evaluating its diagnostic potential on PCa.

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Identification of new early biomarkers of Diabetic Nephropathy using proteomic approaches

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Background:
Diabetic nephropathy (DN) is a serious kidney complication of diabetes. The current clinical evidence of DN is microalbuminuria which refers to abnormal amounts of albumin in urine, but it has inadequate specificity. Over the years, there have been strong efforts to find next generation of early DN onset biomarkers in order to identify and treat at risk patients. However, retrospective clinical cohorts are long and difficult to recruit. We propose an alternative strategy to retrospective cohort recruitment by designing an original clinical study. Then new early biomarkers were identified for early diabetic nephropathy onset detection using a combined proteomics and networks methodology.

Methods:
We have selected a subpopulation of type 1 diabetic patients (N=12) with low albuminuria but at risk for developing diabetic nephropathy. The risk of developing DN was predicted by monitoring urinary albumin before and after a controlled exercise test. A control cohort of normoalbuminuric type 1 diabetic patients was also enrolled (N=14). By 2D-gel electrophoresis, we compared the urinary proteomes of patients from both diabetic cohorts, at risk of developing DN and control, before and after exercise.

Results:
The urine biomarkers were identified by combining urine with fine signal/noise, statistic and biological network studies. One hundred fifty seven distinct protein spots were differential expressed corresponding to 70 identified proteins. Functional analysis of this protein set showed that most of the identified proteins were found belonging to specific molecular networks. The diagnostic potential of these proteins was crossvalidated by western blotting on human samples.

Conclusions:
Differential proteins were identify to enable detection of diabetic patient at risk to develop nephropathy. The nature and physiological function of candidate biomarkers allows better understanding of the molecular mechanisms of DN onset. These findings open the way of early DN patients stratification and monitoring treatment.

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Identification of a novel translation machinery regulating NF1-associated tumors by affinity purification and SWATH (AP-SWATH).

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Background
Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that predisposes individuals to developing benign neurofibromas and malignant peripheral nerve sheath tumors (MPNST). The mechanism of NF1-tumoigenesis or the curatives have not been established. Using iPEACH (Kobayashi et al, MCP 2013), we previously found that translationally controlled tumor protein (TCTP) is a novel biological target for NF1-associated tumors (Kobayashi et al. JBC 2014). In this study, to examine roles of TCTP in NF1-associated tumors in detail, we identified TCTP-interacting proteins by affinity purification using FLAG-tag system and SWATH, and studied their biological function.
Methods
For AP analysis, Flag-TCTP were overexpressed into NF1-deficient MPNST(sNF96.2) cells, and the Flag-TCTP binding proteins immunoprecipitated were subjected to SWATH analysis(Ekisigent/nanoLC-SCIEX/TripleTOF6600). For validation, Flag–target protein plasmids and the TCTP-siRNA were co-transfected, and their binding proteins were quantified by SWATH. For further functional analysis, the target protein specific siRNAs were transfected into MPNST cells, and the total proteins were quantified by SWATH. For biological validation, azidohomoalanine-fluorescent-staining was performed using MPNST cells.
Results
The AP-SWATH analysis identified 113 TCTP-binding proteins with high confidence, and revealed that TCTP mainly interacts with the proteins related to protein synthesis, especially elongation factor complex components, including EF1A1, EF1A2, EF1B, EF1D, EF1G and VARS, in MPNST cells. Interestingly, TCTP preferentially binds to EF1A2, found only in neural and skeletal-muscle cells, rather than EF1A1 despite the high homologies in their sequences, and contributes to the novel activation of EF1A2-denpendent translation by mediating the interaction between EF1A2 and other elongation factors in NF1-deficient tumor cells. EF1A2-knockdown significantly down-regulated the molecules related to protein-translation, and caused the suppression of growth/translation in the cells
Conclusions
We here demonstrate that a novel translational machinery via TCTP-EF1A2 interaction regulating the NF1-associated tumor growth, which could be a therapeutic target for NF1-associated tumors.

Keywords
NF1, TCTP, AP-SWATH, translation

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Automated High-Throughput Sample Preparation Protocols for LC-MS/MS Analysis of Glyco-Omics

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Glycosylation is one of the most important post-translational modification (PTM) of proteins and contains various biological significances. Carbohydrate-protein interactions are involved in many cellular events such as protein localization, protein-protein interaction, immune responses, cell division, tumor immunology and cell signaling. Profiling of glycoprotein is an essential starting point to investigate the cellular events.
The preparation of glyco-proteome samples involves delicate target enrichment and purification steps. These make difficult for processing large number of manual sample treatment, which are usually required in clinical biomarker discovery.
In this presentation, we show three automated Glyco-Omics sample preparation workflows for a 96-well plate liquid handling robotic system. The protocols are based on the filter-aided capture and elution method. First, we have constructed a method to capture glycopeptides using ConA and WGA lectins with 30-kDa membrane filters. Subsequently, the extracted glycopeptides are desalted by C18 clean-up process. Secondly, we have developed a method to extract N-glycan and o-glycopeptide at the one batch experiment. Thirdly, using the SNA lectin and the filters was building a way to extract glycolipid. All of these processes were automated using a liquid handling and a vacuum system. These protocols are efficiently applied for biomarker discovery candidates by limited protein amount of variety of clinical samples, so it is likely to be widely used in diagnosing and treating diseases in the future.

Keywords : Glycosylation, Glyco-Omics, Glycolipid, Automation, Clinical Sample

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PROTEOMIC AND FUNCTIONAL ANALYSES OF

NBDHEX TARGETS IN GIARDIA DUODENALIS

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Background:
Giardiasis affects one billion people worldwide. Treatment relies only on a restricted armamentarium of drugs. The disease burden and the increase in treatment failure highlight the need for novel, safe and well characterized drug options.
The antitumoral compound NBDHEX is effective in vitro against Giardia trophozoites and inhibits glycerol-3-phosphate dehydrogenase. Aim of this work was to search for NBDHEX protein targets and define NBDHEX mechanism(s) of action.
Methods:
A proteomic analysis was performed to select and detect NBDHEX modified proteins in treated trophozoites. In silico structural analysis, intracellular localization, and functional assays were performed to evaluate drug effects on the identified targets.
Results:
A small set of proteins was found covalently bound to NBDHEX at specific cysteine residues. These proteins include metabolic enzymes, e.g. thioredoxin reductase (gTrxR), as well as elongation factor 1B-γ (gEF1Bγ), and structural proteins, e.g. α-tubulin. We showed that NBDHEX in vitro binds to recombinant gEF1Bγ and gTrxR, leading to drug modification of gTrxR catalytic cysteines, with concomitant inhibition of disulphide reductase, and increased NADPH oxidase activity. Moreover, gTrxR nitroreduces NBDHEX, likely via conversion into reactive intermediates, which expand its toxicity.
Conclusions:
Our results indicate that NBDHEX reacts with multiple targets, hampering the functions of at least some of them. The described NBDHEX pleiotropic action accounts for its antigiardial activity and encourages the use of this drug as a promising alternative for the future treatment of giardiasis.
Keywords:
Giardia, NBDHEX, Thioredoxin reductase, Elongation factor 1B-γ

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A dynamic picture of the ubiquitinome upon proteasome inactivation.

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The 26S proteasome is a large protein complex, which degrades unneeded and damaged proteins that are tagged with the small protein ubiquitin. Malfunctioning of the ubiquitin–proteasome system (UPS) has been implicated in diseases such as cancer and neurodegenerative disorders. Here, we dissect the molecular mechanisms of the proteasome by monitoring the dynamic global proteome and ubiquitinome (all ubiquitinated proteins).

The proteome and ubiquitinome are analyzed upon proteasome inactivation by selective RNAi knockdown of subunits using a SILAC and diGly peptide enrichment approach in mammalian and Drosophila cells. Both the diGly peptide enrichment protocol and the decision tree on the Orbitrap Fusion are modified to achieve a deeper coverage of the ubiquitinome.

The proteome is hugely affected when the proteasome is dysfunctional. After inactivation by chemical inhibitors, the abundances of several 100s of proteins are upregulated, including proteins involved in stress response, cell cycle regulation, apoptosis and the UPS itself. Similar effects are observed after inactivation of the proteasome by selective RNAi knockdown of various selected subunits. In addition, the ubiquitinome is dramatically remodeled upon proteasome inactivation. Although the far majority of proteins become increasingly ubiquitinated, several proteins are surprisingly identified with simultaneous increased and decreased ubiquitination on different lysine residues. Proteomic analysis of cells in which one of the three proteasome associated DUBs is depleted reveals major differences and suggests different functions and/or specificities for these enzymes. Finally, using a powerful combination of extensive peptide fractionation, a more economical use of diGly antibody beads and a highly efficient Orbitrap decision tree in which least intense peptides are fragmented first, we can now identify >23,000 diGly peptides in a single sample routinely. Analysis of the dynamic proteome and deep ubiquitinome after perturbation of proteasome function by depletion of specific subunits gives detailed insight into the regulatory mechanisms of this cellular machinery.

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Increasing dynamic range and sample insight for Top-Down Proteoform Profling analysis.

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Background :

Top-Down approaches are gaining in popularity as they detect proteoforms which cannot be distinguished once they have been digested. In such approaches, the increased complexity of the MS signal resulting from the protein's charge state distribution reduces the proportion of proteoform that can be fragmented in a bottom-up like data-dependent MS/MS experiment. In this study, we are developing new precursor selection strategies to increase the information level which can be obtained from an intact protein LC-MS/MS or CE MS/MS experiment.

Methods:

The complexity of protein mixtures constitute a challenge for precursor selection algorithms : if the most intense proteins are efficiently selected, the overlap of charge state envelopes and the difficulty to perform an efficient "on-the-fly" deconvolution for the lowest abundance proteins makes it challenging to pick the lowest abundance compounds once the most intense have been excluded.
By using a Scheduled Precursor List approach, we have first established that the dynamic range covered by the MS intensities of successfully fragmented proteoforms (MS/MS leading to identification) could jump from 1 to close to 3 orders of magnitude. We have then evaluated the robustness of a newly implemented online deconvolution algorithm, the purpose of which is to force the system to select only a specified number of charge states per charge state envelope. The number of identifications, as well as the distribution of the intensities for the selected parent ions are used as evaluation metrics. The online deconvolution algorithm efficiency is compared with a more advanced offline SPL generation script, which selects only the 2 most intense charge states for each proteoform.

Results:

the first results show that the number of proteoforms that could be identified from an E.Coli sample could be increased by a factor > 2.

Keywords :

Top-Down
MS/MS parent ion selection

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Sensitivity, specificity and accuracy of the targeted and shotgun mass-spectrometry approaches

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Background
There are about 2,579 missing proteins (NeXtProt PE2,3,4) in the human proteome. They have not been detected in any biological sample using existing experimental methods. They may be not translated or ultralow-copied, so existing methods could not be used to detect them. In this work we compared existing proteomic mass spectromic methods.

Methods
Targeted (Selected reaction monitoring, SRM) and panoramic (Shotgun LC-MS/MS) mass spectrometric methods were compared by sensitivity, specificity and accuracy similarly to the FDA diagnostic methods (FDA, 2007). Transciptomic analysis of the same sample (Zgoda et al., 2013, Ponomarenko et al., 2014; Poverennaya et al., 2016), or calibration standard UPS2 set (UPS2 set, Sigma Aldrich) were used as a golden standard. Proteomic analysis results were compared with the golden standard for true and false identifications revealing.

Results
Mass spectromic methods were evaluated using FDA indicators. Sensitivity of SRM in pure UPS2 solution is 92% (44 proteins from 48 detected), in biological matrix (E.coli или human plasma) it decreases to 63%. Shotgun LC-MS/MS reveals 23 proteins in the pure UPS2 solution and 11 proteins against E.Coli background. In HepG2 cell line and liver tissue shotgun LC-MS/MS demonstrated sensitivity 6%, and SRM - 35% with the transcriptome golden standard. Both methods have high specificity (more than 90%), but the accuracy is only 57% for SRM and 19% for shotgun mass-spectrometry.

Conclusions
Using indicators of sensitivity, specificity and accuracy for proteomic methods demonstrated, that proteins are "missing" in the sample due to different reasons, for example, chemical noise from other molecules in the biological matrix. Thus, biological matrix Significantly affects the list of detected proteins.

Keywords mass-spectrometry, selected reaction monitoring, shotgun MS, accuracy, sensitivity, specificity

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The PRIDE database: A proteomics data “gold mine” at your disposal

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Background
The PRoteomics IDEntifications (PRIDE) database at the European Bioinformatics Institute (EMBL-EBI, http://www.ebi.ac.uk/pride), as part of the ProteomeXchange Consortium, is the world leading public resource dedicated to storing and disseminating mass spectrometry proteomics data, continuously produced by the global proteomics community. Thanks, among other efforts, to the success of PRIDE and ProteomeXchange, the proteomics community is now widely embracing open data policies, an opposite scenario to the situation just a few years ago.
Methods
This plethora of public data is being increasingly reused by the research community, e.g. in proteogenomics approaches, to build spectral libraries, for tool benchmarking or in innovative meta-analysis studies, among other applications. In this context, we aim that PRIDE becomes a Hub for proteomics data for all life scientists, by disseminating proteomics data into highly popular EMBL-EBI resources such as UniProt, Ensembl and the Expression Atlas.
Results
By April 2017, PRIDE stored over 5,100 datasets which amounted to 300 TB of data, from more than 1,000 different species. Here we will showcase several high-profile examples of PRIDE data reuse efforts both performed by our team and by third parties. For instance, we have started to perform quantitative data reanalysis of suitable public datasets, to be made available in the Expression Atlas at EMBL-EBI. There, we hope to achieve an integrated representation of protein and gene expression information, which will be available for different human tissues and model organisms.
Conclusions
Data sharing in mass spectrometry based proteomics in resources like PRIDE is now a common scientific practise. This situation, unprecedented in the field, opens a plethora of opportunities for data scientists.
Keywords: database, data mining, proteogenomics, reanalysis, spectral libraries

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Defining the dynamic pro-inflammatory response of human monocyte-derived macrophages using the hyperLOPIT spatial proteomics approach.

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Background
The localization of proteins within subcellular, membrane-bound structures is a defining feature of eukaryotic cells. Protein trafficking and translocation between organelles underlie many functional processes and signalling cascades, and aberrant protein localization is a hallmark of many diseases. Toll-like receptor (TLR) induced secretion of pro-inflammatory cytokines from macrophages is a key mechanism of the innate immune response, which involves tightly regulated processes across different intracellular trafficking routes. Here we apply a sophisticated spatial proteomics approach to investigate the cellular machinery implicated in TLR activation, and to identify global changes that occur during polarization of pro-inflammatory macrophages.
Method
We have recently developed hyperLOPIT (hyperplexed-Localisation of Organelle Proteins by Isotope Tagging), a high-resolution spatial proteomics method for the precise “mapping” of thousands of proteins. This method combines biochemical fractionation by density gradient ultracentrifugation, multiplexed tandem-mass-tag (TMT) quantitative proteomics and mass spectrometry. Bespoke machine-learning tools enable the classification of proteins to distinct subcellular niches based on similarity to well-annotated organelle marker proteins. HyperLOPIT was used to identify the steady-state location of thousands of proteins within the human monocytic cell-line THP-1 and evaluate proteomic changes following lipopolysaccharide (LPS) stimulation.
Results
A time-course of LPS-stimulation was used to define global protein abundance changes that occur during monocyte-macrophage differentiation. A single time-point of LPS stimulation was selected for hyperLOPIT evaluation of protein trafficking events. Three biological replicates of unstimulated versus stimulated THP-1 macrophages were analyzed, and over 5,000 proteins were assigned to distinct subcellular locations using the pRoloc analysis platform. Pro-inflammatory markers were identified and several proteins involved in cell migration, adhesion and vesicle trafficking were shown to relocalize following LPS activation.
Conclusions
We have conducted the first spatial proteomics investigation of the pro-inflammatory innate immune response in human macrophages and have revealed a key role for protein relocalization in this process.
Keywords
Spatial proteomics

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Proteome-wide analysis of cysteine oxidation reveals metabolic sensitivity to redox stress

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Reactive oxygen species (ROS) are crucial regulators of biological processes through the oxidative modification of cysteines. ROS play key roles to maintain physiological functions of proteins, and elevated ROS levels are implicated in many diseases. Thus, it is crucial to understand ROS-mediated signalling. Oxidative modifications on cysteines are of low stoichiometry and abundance. Here, we present a new Stable Isotope Cysteine Labelling with Iodoacetamide (SICyLIA) proteomic approach to quantitatively assess cysteine oxidation at proteome-wide sensitivity in cells and tissues.

Cells/tissues were lysed in the presence of stable isotope-labelled (heavy) or light iodoacetamide to swiftly alkylate free cysteine thiols. 150 µg of differentially labelled proteins were mixed using a label-swap replication strategy. Reversibly oxidised thiols were reduced, alkylated using N-ethylmaleimide, and proteins digested with trypsin. Aliquot of the digested samples were modified with dimethyl labelling and used for protein quantification to normalise oxidised cysteine quantification during data analysis. All peptides were fractionated using high resolution high pH reverse phase chromatography and analysed on a Q-Exactive HF. Data were processed with MaxQuant and analysed with Perseus. We report results obtained from four biological replicates.

With SICyLIA we performed comparative analysis of cysteine oxidation of cells and tissues wild-type or knock-out for a gene regulating oxidative stress. We quantified 18,026 (cells) and 10,742 (tissue) cysteine-containing peptides. Of those, 8,689 (cells) and 3,398 (tissue) peptides, which belong to 3,368 (cells), 2,103 (tissue) proteins, were accurately quantified in all replicate experiments. We pinpointed subsets of cysteines with increased oxidation levels in knock-out samples.

SICyLIA informs on all possible cysteine oxidative modifications and achieves proteome-wide sensitivity with unprecedented depth without using enrichment steps. The applicability of this method to cells and primary tissues holds promise for future applications to study oxidative event associated with diseases in patient samples.

Oxidative stress, ROS, cysteine oxidation quantitation, Q-Exactive HF.

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Evolutionary plasticity of neuroplastin basigin and embigin associated complexes with calcium and monocarboxylate transporters

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The single span transmembrane proteins basigin, embigin and neuroplastin are part of a subfamily of immunoglobulin (Ig) domain containing proteins that is widely expressed in mammalian tissue. All three proteins share at least two extracellular Ig domains and a short cytoplasmic C-terminal domain with primary sequences that are overall poorly conserved among species. We investigated the function of these Ig-domain containing proteins by combining affinity purification from native tissue with high-resolution quantitative mass spectrometry. We found exclusive association of neuroplastin with the plasma membrane calcium ATPases 1-4 (PMCA1-4) and of embigin with the monocarboxylate transporter 1 (MCT1) in rat/mouse. In contrast, basigin was detected in complexes with both, MCTs, and PMCAs. In rat brain, this dual binding profile was dependent on stage of postnatal developmental.
Subsequent functional analyses identified neuroplastin and basigin as auxiliary subunits of PMCAs and/or MCTs; in fact, both transporters required co-assembly with the two Ig proteins for stability and proper trafficking. Interestingly, basigin or neuroplastin were able to mutually replace each other in native tissue as observed upon genetic deletion of sh-RNA mediated protein knockdown. Further analyses showed that distribution patterns and specifications of neuroplastin and basigin exhibit marked variations among tissues and species. Most notable, in human erythrocytes, basigin rather than neuroplastin can be seen as the main auxiliary subunit in the complex formation with PMCA and MCT. In this context it is interesting to note that the extracellular part of basigin interacts with RH5 (reticulocyte-binding protein homologue 5), which is central to all malaria inducing Plasmodium falciparum species.
Together, our proteomic approach revealed the importance of the Ig proteins basigin, embigin and neuroplastin for the cell physiology of PMCAs and MCTs.

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Detection of low abundant HPV16-derived HLA-A2 epitopes on the tumor cell surface by mass spectrometry

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Background
Detailed knowledge about human papillomavirus (HPV) CD8+ T cell epitopes that are naturally presented on HPV-infected and -transformed cells is essential for rational design of therapeutic HPV vaccines. To date, target HPV16 T cell epitopes have mostly been determined by indirect biological methods. We aimed to directly identify low-abundant human leukocyte antigen (HLA)-A2-restricted HPV16 epitopes on the surface of HPV16-transformed cells using a nano-UPLC-ESI-MS methodology employing selected reaction monitoring (SRM).

Methods
To select epitopes for MS detection on the cell surface, in silico epitope predictions from the HPV16 E6 and E7 proteins were performed with several web-based prediction algorithms. Predicted binders were tested for actual HLA-A2 binding in competition-based cellular binding assays. HLA-A2-peptide complexes were immunoprecipitated (IP) from HPV16-transformed cells. Peptides were dissociated from complexes and purified. The presence of verified binding peptides was analyzed by nano-UPLC-ESI-MS. Resulting spectra were compared to reference spectra of synthetically produced verified binding peptides.

Results
Out of the HPV16-derived HLA-A2 peptides predicted to be binders, binding of 31 peptides could be verified in the cellular binding assays. We identified several novel HPV16 HLA-A2 binding peptides. In total, 17 non-cysteine containing HPV16 peptides were monitored by nano-UPLC-ESI-MS in the IP samples. Only the HPV16 E711-19 peptide had previously been reported to be presented on HPV16-positive cell lines and tumor samples by MS. We identified the E711-19 peptide and 13 other HPV16-derived epitopes on the surface of HPV16-transformed cells.

Conclusion
Our strategy for direct identification of low abundant HPV16-derived epitopes on the cell surface resulted in identification of 14 HLA-A2 epitopes, 13 have never been detected by MS before. The approach is currently being extended to identification of HPV16 HLA-A3- and HLA-A24-restricted epitopes. In general, directly identified epitopes form a solid base for immunotherapy design.

Keywords: T cell epitope, HLA-A2, human papillomavirus (HPV), SRM

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A draft proteome map of the Ginseng(Panax Ginseng C. A Meyer): Mass spectrometry-based proteomic approach

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Ginseng (Panax ginseng C. A. Meyer) is well known medicinal crop, routinely used in Eastern countries. Because of its health promotion effects against several diseases, several research groups across the globe are working on this wonder plant. However, most of the ginseng research has been focused on analyzing the medicinal properties of ginseng root and identification of medicinal compounds like ginsenoside. The information regarding the ginseng proteome remained largely elusive. Here, an attempt was made to generate the draft proteome map of ginseng using shotgun proteomics approach coupled with Q-Exactive high resolution mass spectrometer. Total proteins, isolated from leaves, shoots, roots, and fruits, in four replicates were utilized for proteome analysis. As the ginseng genome has not been sequenced yet, an in-house developed RNA-Seq database (PacBio) was used to increase the protein identification. A total of 34,563 peptides, matching with 4,556 unique proteins were identified using Andromeda, integrated with MaxQuant software. On applying stringent criteria for valid protein identification (unique peptides ≥ 2 and presence of a protein in all the four replicate) using Perseus software, a total of 1,728 high-confidence proteins were identified. Label-free quantitative proteome analysis led to the identification of 221, 210, 153, and 32 leaf, shoot, root and fruit specific proteins respectively. Functional annotation of the commonly identified proteins using KEGG and Panther web based software showed these to be with majorly primary metabolism, secondary metabolism, ribosomes, glycolysis/glucogenesis and protein processing in endoplasmic reticulum, among others. Taken together, this is the first attempt to generate the draft proteome map of ginseng using high-resolution mass spectrometry and the results obtained provide new insights into the protein complement of ginseng.

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Proteomic profiling of secretomes from M.tb-stimulated, PUFA-treated, ex vivo PBMCs in TB-IRIS

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Background
Tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) is an inflammatory disorder which affects 8-54% of TB-HIV co-infected patients. Its pathogenesis remains unclear, and no specific treatment is currently available. N-3 polyunsaturated fatty acids (PUFA) have safely and successfully been used in the treatment of other inflammatory diseases, and we hypothesize that they will have a beneficial anti-inflammatory effect on TB-IRIS patient-derived immune cells.

Methods
Peripheral blood mononuclear cells (PBMCs) were obtained from eight healthy donors and five TB-IRIS patients, re-stimulated ex vivo with whole M.tb lysate, and treated with n-3 PUFA (EPA and DHA) for 24 h. The secretome was prepared via choloroform/methanol precipitation and overnight Trypsin digestion, and investigated via mass spectrometry-based shotgun proteomics. MaxQuant was used for protein identification and Perseus for statistical analysis. We also investigated the effects of prednisone (the current treatment for TB-IRIS) on the proteome of M.tb-stimulated PBMCs.

Results
Stimulation with M.tb shifted the secretome of healthy PBMCs towards an inflammatory state, and this was relieved by treatment with EPA/DHA via up-regulation of redox pathways. Preliminary results from TB-IRIS patient-derived PBMCs show the same trend. These promising early results suggest the potential benefits of n-3 PUFA dietary supplementation for patients with TB-IRIS.

Conclusions
With these encouraging results, we plan to increase the sample size to confirm findings, and hope to include n-3 PUFA supplementation in the next clinical trial on patients with TB-IRIS.

Keywords
Polyunsaturated fatty acids, secretome, TB-IRIS, peripheral blood mononuclear cells, Mycobacterium tuberculosis

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A mass spectrometry approach for the identification and localization of lysozyme modifications by acrolein

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Background: Lipids containing polyunsaturated fatty acids are primary targets of oxidation, producing reactive products including a variety of short-chain aldehydes. These reactive species can covalently modify proteins in a process called lipoxidation, and these oxidative post-translational modifications influence cell behaviour and can be involved in a range of inflammatory disorders. The exact nature of many of these adducts, and their relationship with cellular effects is still unclear. There is a need to identify the broad range of modifications that are generated in lipoxidation, and develop sensitive mass spectrometry (MS) methods for the sensitive and selective identification of these adducts in complex biological systems.
Methods: Model peptides and proteins was used to investigate the formation of short-chain aldehyde-containing lipoxidation products. The target was modified with the exemplar alkenal acrolein, the adducts formed stabilized by reduction with sodium cyanoborohydride, and the adducts identified using MS and MSMS to analyse the intact protein and tryptic digests.
Results: Analysis of modified peptides identified a range of different adducts including novel structures, which were identified through the MSMS data. Modification of lysozyme demonstrated that multiple sites (up to 8) could be modified. Analysis of tryptic digests allowed the localization of the adducts to specific amino acid residues and the identification of amino acid-specific fragmentations, and modifications of human serum albumin were also identified as potential in vivo markers of lipoxidation.
Conclusions: MS methods provide a powerful tool for the identification and localization of lipoxidation modifications of proteins and determination of their structures. Specific fragmentation products were identified that could be used for the selective detection of modifications in more complex samples. Quantitative information could be gained on extent of modification of individual sites, and for the protein overall. Further aldehydes and more clinically relevant samples are being investigated.

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Proteomics approach reveals the roles of mitochondria in NLRP3 inflammasome activation in Nasopharyngeal Carcinoma

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Inflammasomes are inducible signaling complexes that can be activated in response to cellular stress in tumor microenvironment. Upon stimulation, pattern recognition receptors NLRP3, AIM2 and RIG-I will be recruited to ASC, a key platform protein for inflammasome activation, resulting in formation of speck-like particles triggering caspase 1 activation which promotes the secretion of proinflammatory cytokine IL-1β. Nasopharyngeal carcinoma (NPC) is a cancer prominent in Taiwanese population. We have examined the NLRP3 inflammasome activation in NPC. However, the mechanisms regulating NLRP3 activation in NPC it is not fully clear. To reveal the difference between the speck (+) and speck (-) cells, we used a ASC-GFP-expressing NPC-HK1 cells, which can be induced by the therapeutic drug cisplatin to activate NLRP3 inflammasomes (speck (+)) cells. As noted that the speck-like particles were detected in some but not all treated cells. Approximately 2.5% cells treated with cisplatin formed specks. We then isolated the cells with or without speck by flow cytometry, followed by proteomic analysis (iTRAQ LC-MS/MS). A total of 634 proteins were enriched (overexpressed) in speck (+) NPC-HK1-ASC-GFP cells after cisplatin stimulation from duplicated experiments. Computational prediction of protein functions revealed that more than 30% proteins enriched from speck (+) cells are play roles in mitochondria. Additionally, GeneGo process network of these enriched proteins are mainly participated in two biological pathways: oxidative phosphorylation and ubiquinone metabolism, suggesting that mitochondria is participated in NLRP3 inflammasome activation. The electron transport chain (ETC) in the mitochondria inner membrane is the major site of oxidative phsophorylation and is also the main source of cellular ROS. We demonstrated increased mitochondrial ROS production are detected in speck (+) NPC-HK1-ASC-GFP cells after cisplatin stimulation. Together, these results provide a new direction for studying the mitochondrial roles involved in NLRP3-ASC speck formation and drug development for cancer through inflammation modulation.

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Effect of intestinal flora on drugs, glucose and lipid metabolism in mouse

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Background
Recent studies suggest that antibiotics have multiple effects on host physiology, including drug, glucose and lipid metabolism. The dysbiosis is known to be associated with various host physiologies. The purpose of the present study was to clarify the effect and regulatory mechanism of dysbiosis induced by antibiotics on the pharmacokinetics of drugs, and glucose and lipid metabolism.
Method
Mice administered orally with non-absorbable antibiotics (vancomycin and polymyxin B) for 5 days and germ-free (GF) mice were used as dysbiosis models, and the protein expression levels in the dysbiosis mice tissues were comprehensively determined by SWATH-MS and targeted proteomics.
Results
In both of the mouse models, the liver expression levels of Cyp2b10 and Cyp3a11, drug-metabolizing enzymes, were markedly decreased and Cyp2b activity in the liver was also decreased. For drug transporter, Oatp1a1 was decreased in the liver and kidney of GF mice, and Bcrp1 was decreased in the liver of both models. It was expected that intestinal bacteria-produced secondary bile acids had an influence on drug, glucose and lipid metabolism. In antibiotic-treated mice, the secondary bile acid producing bacteria in the feces and the concentrations of secondary bile acids (lithocholic acid and deoxycholic acid) in the liver were reduced, and serum glucose and triglyceride levels were also decreased. In addition, the serum glucose and triglyceride levels were recovered by co-administration of the secondary bile acids with antibiotics. With respect to drug metabolism, expression levels of Cyp2b10, Cyp3a25 and Cyp51a1 in the liver were changed by the alteration in hepatic secondary bile acid concentrations.
Conclusions
The present results suggest that dysbiosis induced by short-term antibiotics administration affect the efficacy or occurrence of adverse effect of drugs. Furthermore, bacteria-produced secondary bile acids modulate the expression levels of Cyp enzymes in the liver, and serum glucose and triglyceride levels.

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The forgotten proteome – approaches for the identification of short open reading frame encoded peptides

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Background
The recent discovery of an increasing number of small open reading frames (sORF) creates the need for suitable analytical technologies for the comprehensive identification of the corresponding gene products (sORF encoded peptides, SEP). We evaluated different analytical approaches that allow for the simultaneous analysis of widest parts of the proteome together with the predicted SEP.

Methods
Model organisms such as the archeon Methanosarcina mazei and C. elegans were analyzed by various approaches. Methodologies for the isolation of SEP using different extraction conditions, the depletion of higher mass proteins (> 10kDa) by means of ultrafiltration or GelFree-open tube electrophoresis and the separation by 2D-LC MS utilizing a high/low pH reversed phase LC separation scheme were tested.

Results
The combination of a semi-top down approach and a 2D-LC-MS based bottom up analysis provided the highest proteome coverage for M. mazei reached so far. In addition, 28 formerly unidentified SEP could be identified, which was possible by adaption of the criteria for the interpretation of single peptide MS/MS identifications. The use of novel extraction procedures and the avoidance of ultrafiltration steps were shown to improve detection of SEP. Interaction partners of several SEP could be detected, providing hints for the function of these peptides.

Conclusions
Due to neglecting sORF during genome annotation and the bias of classical proteomics approaches towards proteins larger than 10 kDa, SEP represent a yet forgotten part of the proteome. However, the first important biological functions of SEP have been elucidated, even in human cells, showing that the adaption of analytical approaches for their identification, quantification and characterization bears a high potential to shed light on this “dark” proteome.

Keywords
2D-LC MS, small open reading frame, peptidomics, top-down proteomics

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Refining Treatment Recommendations for Lymph-Node-Negative Breast-Cancer Patients using Novel Protein-Based Prognostic Signature: The OncoMasTR Assay

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Background
Many women with lymph-node-negative breast-cancer are unnecessarily treated with chemotherapy. This highlights the importance of accurately predicting patient prognosis. We previously identified a novel prognostic signature, termed OncoMasTR, encompassing a set of master transcriptional regulators, linked to cell proliferation, together with the key senescence factor CDKN2A (Lanigan et al. 2015).When assessed at the mRNA level, OncoMasTR predicted recurrence risk for lymph-node-negative breast-cancer better than current multigene prognostic assays, particularly in estrogen receptor-positive patients. OncoMasTR accurately classifies over 60% of patients as 'low-risk', an improvement on existing assays, potentially reducing overtreatment in early-stage patients. Here, we describe the ongoing validation of the OncoMasTR signature at the protein level.

Methods
We validated multiple commercial antibodies against proteins within the OncoMasTR signature. CRISPR/Cas9 knockout cell lines were used as controls for Western blotting and immunohistochemistry (IHC), the latter being accomplished using formalin-fixed paraffin embedded (FFPE) cell pellets. Validated antibodies were further optimised for IHC using full-faced sections of breast-cancer tissue. IHC was performed on tissue microarrays (tissue microarraysTMAs) containing repeated cores from several hundred breast-cancer patients. Visiopharm's Oncotopix algorithm was used for image analysis, with stained tissues verified manually by an expert pathologist. Image analysis data was combined with clinical data and survival analysis was carried out using R. Suitable thresholds were identified to differentiate low/high expression of individual markers. Multivariate analysis is used to determine the optimal combination of biomarkers for stratification of risk of recurrence.

Results
We confirmed utility of the OncoMasTR panel at the protein level and demonstrated the prognostic ability of single markers. We are working on combinations of markers to optimise performance, with these analyses ongoing.

Conclusions
The study takes advantage of key regulators of cellular proliferation and senescence to improve prognosis prediction in early-stage breast-cancer.

Keywords
Biomarker discovery – Immunohistochemistry - Personalised medicine – Tissue microarrays

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Novel hypoxia-driven pro-angiogenic mechanisms unveiled by secretomics analysis of mammary CAFs

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Background: Hypoxia correlates with abnormal angiogenesis, increased risk of metastasis and poor prognosis in breast cancer. Cancer-associated fibroblasts (CAFs) are an abundant stromal component of breast tumours. Yet, their role in angiogenesis under hypoxic conditions is poorly characterised. Methods: We combined human primary endothelial cells (ECs), patient-derived CAFs and a 3D angiogenesis assay with SILAC-based MS to assess how the hypoxic environment remodel the CAF proteome and secretome to promote angiogenesis. Results: We found that the secretome of hypoxic CAFs enhances angiogenesis via VEGFA-dependent and -independent mechanisms. Using MS, we ascertain that hypoxic CAFs increased the secretion of pro-angiogenic proteins whilst down-regulating anti-angiogenic ones. Additionally, we identified proteins that have not been previously related to hypoxia or functionally characterized. Amongst those, a leucine-rich repeat containing (LRRC) protein and an antisense protein (AS) were highly up-regulated in hypoxic CAFs. Strikingly, these two secreted proteins enhance endothelial cell (EC) sprouting and migration. LRRC is a novel HIF1α responsive gene induced by hypoxia in different fibroblasts but not in cancer or ECs. Concordantly, LRRC is expressed specifically in the stroma of human and murine breast cancers in vivo. Using a loss of function approach combined with MS secretomic analysis, we found that LRRC regulates the levels of different pro-angiogenic proteins secreted by CAFs, which in turn favour vascular abnormalities. AS expression in vivo occurs in both cancer and stromal cells and its hypoxia-induced levels are controlled post-transcriptionally. MS secretomics analysis showed that AS regulates the levels of VEGFA, the most potent pro-angiogenic factor, and the defects in EC sprouting induced by the silencing of AS in CAFs are rescued via addition of VEGFA. Conclusion: We discovered two novel hypoxia-induced secreted factors derived from CAFs. Our findings can provide new avenues to target the stroma to normalise the dysfunctional vasculature of advanced hypoxic tumours.

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Native peptidomics: Workflow of discovering biomarker for disease from human body fluids.

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Introduction and Objectives

Peptidomics is to comprehensively study endogenous peptides processed from precursor proteins. Endogenous or native peptides may have functions as hormones and other intermediating factors or may have no significant functions as those processed to produce or degradate the functional peptides. Furthermore, native peptides thought to refer the internal body situation, such kind of disease and also human metabolome.
In this study, we established the workflow for identify the native peptide from human urine.

Methods

Human urine samples were obtained from 28 healthy volunteers. The urine was separated with Molecular Weight Cut Off filter and native peptides were precipitated by acetonitrile. Native peptides were purified with C18 spin columns and analyzed by Fusion mass spectrometry by HCD - ETD mixed method. Peptides were analyzed by de novo with wide FDR and strict FDR with >1%.

Results and Discussion

The current analysis showed identification of more than 10,000 native peptides by de novo analysis without any filtration. On the other hand, about 300 and 600 native peptides were identified with strict and wide FDR respectively. Interestingly, these native peptides were assigned to proteins which is related to famous proteins processed and released to body fluids. This result indicated that native peptides could be detectable by this workflow. Furthermore, unique native peptide will be useful marker compare between healthy volunteer and disease patients.

Conclusion

We established the workflow for identify the native peptides from human urine. Some of native peptides assigned to proteins indicated that processed by enzyme and released to body fluids. These data can be helpful to discover the biomarker from native peptides in the future.

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Serving protein interaction data to the community – the HUPO-PSI Molecular Interaction Workgroup.

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Proteins do not function in isolation in living cells but form interaction networks with other proteins, small molecules, lipids, carbohydrates and nucleic acids. Understanding the structure and dynamics of protein networks helps to unravel complex biological processes and to chart the progression of genetic diseases. The Molecular Interaction (MI) workgroup of the HUPO-PSI have worked for 15 years to produce data exchange formats, controlled vocabularies for describing the data and tools to visualise and analyse interaction networks. The PSICQUIC webservice provides 150 million interaction evidences from 32 groups. PSI-MI XML2.5, and the simpler MITAB format, have been widely adopted by those wishing to download and access interaction data. However, new use cases and data types mean that we need to extend these formats to fully meet the needs of the community. XML3.0 enables the description of specialised use cases, such as allosteric interactions and dynamic data. MI-JSON will enable users to efficiently serve MI data to web pages/visualisation tools. We are looking to extend the existing MITAB format to capture the directionality of an interaction and whether the interaction causes an up- or down-regulation of a subsequent event or process, to enable logical modelling and causal reasoning. A simpler representation of molecule features, such as point mutations, has also been developed. In order for users to develop tools that can take advantages of all these data, a Java library, JAMI, that can read/write to all interaction formats is now available. Finally, the work of the IMEx Consortium is making data available in all these formats will be described and planned updates to PSICQUIC briefly outlined.

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Streamlined and sensitive sample preparation for phosphoproteomics using the EasyPhos workflow

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Cell signalling is a rapidly evolving field, yet due to a variety of technical limitations most researchers still rely on laborious and low-throughput antibody-based approaches. The EasyPhos workflow addresses these challenges, and is sufficiently streamlined to enable analysis of hundreds of phosphoproteomes with minimal measurement time with high reproducibility. Recent improvements to the workflow now ensure high performance in sample-limited conditions, while simultaneously reducing sample preparation time. Elimination of protein precipitation steps reduce opportunities for sample loss and variability. Requirements are as little as 250 µg of protein starting material for a depth of >10,000 quantified phosphopeptides in 2 h of measurement time. EasyPhos is accelerating a wave of new in-depth and in vivo signalling studies in diverse fields including insulin signalling and circadian rhythms in mouse liver, GPCR signalling in the mouse brain, glucose-mediated insulin secretion in insulinoma cells, and embryonic stem cell differentiation.

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Epidermal barrier improvement after Excipial application in atopic xerosis is revealed by non-invasive proteome analysis.

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Background : The stratum corneum plays a crucial role in barrier function and is under investigation in several skin pathologies. The stratum corneum (SC) of atopic xerosis (AX) reveals not only decreased hydration but also mildly impaired barrier function characterized by an increase in Trans Epidermal Water Loss (TEWL), elevated pH values, and an increased turnover rate of the SC consisting of thick layers of smaller-sized corneocytes. The mildly impaired SC functions of AX can be improved by daily repeated applications of effective moisturizers, which are effective in preventing the progression of AX to atopic dermatitis (AD). In a recent study, clinical and biophysical observations were in favour of an improvement of skin surface after 8 daily applications of Excipial (anti-itch foam). In AX the effect of moisturizers in general on the stratum corneum protein composition has not yet been fully analysed.
Methods : Mass spectrometry analysis was performed using protein extracted from stratum corneum collected before and 48h after 8 days of Excipial application.
Results : More than 400 proteins were identified in SC. Several proteins were found significantly modulated after Excipial treatment. We found a significant increase in content of proteins involved in cohesion of the SC and its barrier function, such as Desmoplakin and the Keratin type II cytoskeletal 2 and in regulation of epidermal homeostasis, such as filaggrin-2. In addition, we found two abundant unique peptides of serum albumin slightly but significantly decreased after treatment. Using Luminex technology a slight decrease in serum albumin content was observed (fold: -1.5, $p < 0.05$). Interestingly, albumin was previously described up-regulated in AD.
Conclusions : Taken together, our proteomics data indicate that skin homeostasis and barrier function in AX was improved at the molecular level after 8 days Excipial application.
Keyword : Stratum corneum, barrier function, moisturizers, epidermis

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First direct evidence of cross-linked Aβ dimers in the brains of Alzheimer's disease patients

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Background: Brain-derived amyloid-β (Aβ) dimers are associated with Alzheimer´s disease (AD) and have been shown to impair synapse structure and function, as well as to induce neurofibrillary degeneration. However, the covalent nature of these oligomers remains to be established, being one of the biggest challenges to establish whether these dimers are non-covalent or covalently linked. This issue is relevant as a covalent cross-link would make brain-derived dimers more synaptotoxic and suitable candidates for biomarker development.

Methods: We isolated and targeted cross-linked (CL) Aβ dimers in the brains of AD patients using synthetic CL Aβ dimers as standards, biochemistry tools and state-of-the-art bottom-up and top-down mass spectrometry (MS) approaches.

Results: MS analysis of brain-derived Aβ dimers isolated from AD brains led to the detection of the (H6-K16)$_2$ CL peptide, a peptide specific to CL Aβ dimers and to the identification of the first endogenous CL Aβ dimer, Aβ(1-34)-Aβ(3-43).

Conclusions: Having established the presence of CL Aβ dimers in the brains of patients with AD, our work opens up avenues for establishing new targets and developing novel biomarkers for AD.

Keywords: Alzheimer's disease, amyloid-β, cross-linked dimers, oxidative stress

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OmicsDI RDF

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Background
The Omics Discovery Index (OmicsDI, http://www.omicsdi.org/) provides dataset discovery across a heterogeneous, distributed group of Transcriptomics, Genomics, Proteomics and Metabolomics data resources. It provides a short description of every dataset: accession, description, and publication, etc. Based on these metadata, OmicsDI provides extensive search capabilities. Although OmicsDI is an important resource for multi-omics research, it is more valuable to connect with other resources such as UniProt, Ensembl, and PubChem. In the context of 'Linked Open Data', a concept about connecting data independently of the involved biological data types, we chose the Resource Description Framework (RDF) data model in order to link between OmicsDI and other resources.

Methods
We designed an RDF schema for the OmicsDI datasets and implemented a converter from the existing OmicsDI XML format to RDF. The converted RDF files were loaded into a triplestore, which is a data management system for RDF. Although well-known ontologies and controlled vocabularies (CV) were reused to describe concepts and relationships (such as Dublin Core and the PSI-MS CV), we have defined additional ontology terms as needed.

Results
As of October 2016, there were 80,000 public dataset entries accessible through OmicsDI XML, which were converted to RDF. Once the converted RDF files were loaded into a triplestore, we were able to successfully search OmicsDI datasets by using much more complex queries. Since we employed the RDF data model, which is globally used e.g. in federated queries, we are not only able to search for OmicsDI datasets, but also integrate these searches with datasets from other resources such as UniProt, Ensembl and PubChem.

Conclusions
We designed an RDF schema for OmicsDI metadata, and converted from OmicsDI-XML to OmicsDI-RDF. It enables linking of OmicsDI-RDF with other resources.

Keywords
Metadata, OmicsDI, RDF, data integration, multi-omics

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Open-glycomics: An open-access platform for automated glycan identification and quantitation

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Background
Porous graphitised carbon (PGC) LC-MS/MS analysis of glycans can provide structural information such as monosaccharide composition, sequence and branching, which is important for understanding their role in biology. Characterising and quantifying glycans is challenging due to their dynamic range, glycan isomer structural similarity and lack of vendor-neutral, open-access tools for quantifying glycan isomers across LC-MS/MS datasets. Here, a streamlined data acquisition and Skyline-based analysis platform for glycomics was applied to PGC-LC-MS/MS data on a large repertoire of reduced N- and O-glycans released from purified proteins and complex protein mixtures.

Methods
Our data acquisition approach uses PGC to achieve LC separation of glycan structural isomers, allowing characteristic resonance activation CID-MS/MS of each isomer to be acquired in negative ion mode. The fragmentation of a wide-range of glycans was optimised for complete precursor fragmentation and generation of diagnostic ions to allow for discrimination between glycan isomers, providing reproducible and deep characterisation of glycan monosaccharide composition, sequence and branching. Skyline, a software tool commonly used in proteomics, was adapted for automated quantitation of glycans. Diagnostic fragment ions representing glycan structural features including core-fucose and antennae composition were validated and used to automatically discriminate between glycan isomers.

Results
Skyline provided chromatograms of each observed charge-state of all characterised glycans and the corresponding peak areas were integrated automatically from the MS1 data. From a complex mixture, glycan isomers, differing in as little as a single glycosidic bond, were separated, identified and quantified in a single LC-MS/MS run using Skyline with minimal user intervention.

Conclusion
Optimisation of the CID-based fragmentation of released glycans and the adaptation of Skyline to quantify and discriminate between glycan isomers allowed an increase in analysis throughput while maintaining high accuracy. This open-access platform for label-free glycan quantification will improve data sharing and stimulate further method development in glycomics.

Keywords
Glycomics

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Targeted and panoramic views on the transcriptome and proteome of liver tissue and HepG2 cells

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Background. A number of recent studies show that correlation between transcript and protein levels within same sample is very poor. Absence of protein detection can be caused by biological factors as well as sensitivity limitations of proteomic methods (Archakov et al., 2012). We use transcriptomic data as a standard when evaluating of the results of proteomic experiments, since the presence of mRNA indicates the presence of gene expression and, therefore, potential possibility of detecting the corresponding protein in the sample.
Methods. Within the Human Proteome Project we studied the transcriptome and the proteome encoded by genes of chromosome 18 (Poverennaya et al., 2016). We analyzed the cells of the liver tissue and HepG2 cell lines using panoramic analysis methods - RNA sequencing and shotgun LC-MS/MS, along with the directional measurements for quantitative analysis. Information about the transcripts copy number per cell was obtained by qRT-PCR method, while the copy number of protein (corresponding to given transcripts) was obtained by targeted mass-spectrometry (SRM) with use of isotope-labeled peptides as standards.
Results. The study defined the parameters of bioinformatics guidelines of transcriptomic and proteomic data analysis under which the list of detected proteins most closely matches the transcripts found in the same sample. Were calculated sensitivity, specificity and accuracy of various methods of proteomic analysis applicable to the transcriptome of the same sample of biological material, by analogy with the evaluation of diagnostic methods.
Conclusions. Integration of targeted transcriptomic analysis and data obtained by SRM method from the same sample appears to be the most promising solution for detecting so-called “missing” proteins.
Keywords. C-HPP, human proteome, meta-analysis, mRNA, Chromosome 18

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Large scale integration of proteomics and genomics data to enhance rice genome annotation

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Background
Rice is perhaps the most important worldwide crop – providing around 20% of the total global dietary energy. While the current quality of the rice gene model annotation is far from complete, it can be assumed that many of the “official” gene models may not be correct (leading to incorrect protein sequences).

Methods
The proteogenomics approach uses proteomics data to improve gene model annotation, since they can provide evidence that predicted splicing events (including alternative splicing) are translated to a protein product. We have developed a proteogenomic analysis tool, ProteoAnnotator and used it to integrate RNA-seq and proteomics data of rice to enhance its genome annotation by identifying novel splicing events. The results can also be viewed via customised Track Hubs on common gene browser for further investigation.

Results
In this project, 29 rice RNA-seq data sets were collected from European Nucleotide Archive to produce “novel splice junction” and “novel transcript” databases using existing tools (i.e. TopHat and Cufflinks) by using the official rice genome database as reference. These DNA sequences were then translated into a novel protein database. In total, 9 rice proteomic data sets from PRIDE/ProteomeXchange were analysed. They were searched against a combined protein database (novel plus official) using IPeak/ProteoAnnotator for evidence of novel peptides. We found 352 and 329 novel peptides only from the transcript and junction database respectively, while 195 novel peptides were found from both databases. Further analysis shows that 279 of 352 novel peptides from novel transcript database are from all 29 original RNA-seq data sets, which gives strong evidence for the existence of the transcripts they have been matched to.

Conclusions
We have demonstrated the feasibility of using proteogenomics to discover novel peptides, supporting updates to the rice genome annotation.

Keywords
Proteogenomics; Oryza Sativa; Annotation; Bioinformatics

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Functional Protein Networks and Disease: An Affinity Based Approach

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Understanding the orchestration and dynamics of proteins within a tissue or organ is one of the challenges in biology. Combining affinity proteomics quantitative mass-spectrometry, in silico network analysis as well as structural biology we have developed an analytical approach to determine functional constraints in protein networks that drive biological functions and - when hit by mutation - cause disease. The approach allows us to rationalize and hypothesize, how protein network topology and genetic architecture interact to define normal physiological function and give us insights into the molecular mechanisms of disease. We have applied affinity proteomics, genetics and cell biology to interrogate cilia: poorly understood organelles, where defects cause genetic diseases. Two hundred and seventeen tagged human ciliary proteins created a final landscape of 1,319 proteins, 4,905 interactions and 52 complexes. Reverse tagging, repetition of purifications and statistical analyses produced a high-resolution network that reveals organelle- specific interactions and complexes not apparent in larger studies, and links vesicle transport, the cytoskeleton, signaling and ubiquitination to ciliary signaling and proteostasis. We observed sub-complexes in exocyst and intraflagellar transport (IFT) complexes, which we validated biochemically, and by probing structurally predicted, disruptive, genetic variants from ciliary disease patients, defining new ciliopathies. The resulting information has not only offered a comprehensive view on the mechanisms of selective protein transport and protein complex assembly but also suggests novel signaling routes guiding these activities via small GTPases. The approach presents a generic multiscale workflow applicable to the analysis of any cellular pathway or protein network and opens a new field that we define as structural systems biology. This can serve as a basis for elucidating physiological principles as well as to assist molecular medicine and pharmacology.

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Chronic administration of methylmercury induces differential proteome changes in different regions of mammalian brains

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Paresthesia and numbness of the skin are earliest syndromes of MeHg intoxication. Other follow up syndromes include dystaxia, dysarthria and visual impairment. The sequential occurrence of pathological syndromes indicates that different regions of the brain respond differently to MeHg. On the other hand, continue consumption of seafood imposes an increased risk of exposure to MeHg. In rats exposed to 40 µg/kg body weight/day of MeHg for 12 weeks accumulated different amounts of Hg loads in different regions of the brain. With proteomic technologies, we found dramatic changes in proteome of the somatosensory cortex. Specifically, proteins related to glycolysis, ATP production, neurotransmission, and protein synthesis were down-regulated, resulting in a metabolic deficit without observable abnormality phenotypically. In contrary to results obtained from the somatosensory cortex, we found different sets of differentially expressed proteins in the other regions. In the visual cortex, motor cortex and the cerebellum, several proteins involved in synaptic transmission and glycolysis were up-regulated. Similar proteome changes due to MeHg accumulation in the various brain regions of two top marine consumers (ringed seals and polar bears) were also observed. In summary, we found that different regions of the brain showed different proteome changes and this may account for the sequential occurrence of different pathological syndromes in human suffered from acute and increasing MeHg loads.

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Characterization of Data-Independent Acquisition (DIA) capabilities of a Q-TOF instrument for complex proteomics samples

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In the field of MS-based quantitative proteomics, Data Independent Acquisition (DIA) workflows are gaining popularity. While it overcomes the stochastic selection of peptide precursors, it demands high requirements for the instrumental capabilities and the subsequent processing of the data.
The ideal fit of Q-TOF instrument's unique combination of speed, resolution, sensitivity and dynamic range will be demonstrated by analyzing challenging samples consisting of three proteomes in different ratios. The acquired data were processed with the SpectronautTM software suit.

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Quantitation of phosphorylated myelin basic protein in brain tumor tissues using pSRM

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Background
Variations in the protein phosphorylation sites can be associated with the development of pathologies. Most of the existing proteomic quantitative methods have limitations of PTMs detection due to insufficient selectivity and sensitivity. Targeted mass-spectrometry with stable isotopic standards (SIS) is the most suitable method for quantifying of proteins that are phosphorylated at specific sites.
Methods
We phosphorylated MBP in-vitro by MAPK and performed bottom-up analysis of protein products by LTQ-Orbitrap. The obtained data were used to define sequences and pSRM parameters of the most representative proteotypic and phosphorylated peptides. Two corresponding peptide standards with isotope labeled leucine were synthesized. We used these SISs to quantify the total MBP and its phosphorylated form contents in 16 biopsy samples of brain tumors (6 neuroma, 5 astrocytoma, 5 meningoblastoma samples).
Results
We examined content of proteotypic [TAHYGSLPQK] and phospho-[NIVTPR(pT)PPPSQGK] peptides of MBP in the tryptic digests of the tumor tissues. MBP was observed by pSRM in all astrocytoma samples, the phosphorylated peptide was detected in 3 of 5 samples. The proportion of phospho/total MBP content ranged from 12 to 17%. We also detected MBP in 5 of 6 tested neuroma samples and 2 of them were phosphorylated in proportion of 35% to total MBP content. MBP was also observed in 4 meningoblastoma biopsy samples but no phosphorylated sites were detected. The total MBP content in tumor tissues was in range 0,1 fmol – 0,9 pmol per 1µg of total protein.
Conclusion
Using pSRM with SISs we have measured the concentration levels of the protein modified in the specific site with high selectivity and sensitivity in wide concentration range. The applied pSRM approach allows us to analyze the phosphorylation degree of certain proteins in cells, tissues and biological fluids. This study is supported by RSF grant №16-44-03007.
Keywords
Phosphorylation, pSRM, MBP, brain tumor

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Next-generation positional proteomics - navigating substrate degradomes with quantitative digital terminome maps

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Current proteomics workflows for protease substrate discovery mainly focus on N-terminal peptides, and only few approaches for the analysis of protein C-termini in complex proteomes are available. Relative quantification in samples exposed to differential protease activity is achieved by isotopic labels, limiting multiplexing capabilities and forcing rigid study designs. Positional proteomics workflows generally require relatively high amounts of sample and thus prevent terminomics analysis of small total protein quantities available for example from sorted cells or patient biopsies.

We have devised and implemented a next-generation positional proteomics strategy that exploits the concept of label-free quantification and data independent acquisition (DIA). Thereby, we process samples using a slim biochemical protocol that preserves the full peptide-level information contained in the sample. Afterwards, we transform the samples into digital proteome maps using fast, high-resolution LC-MS systems. The resulting maps can be interrogated with high quantitative accuracy for any peptide species of choice using chromatogram libraries. Eventually, targeted data extraction is only limited by the library content and ideally covers all peptides accompanying a proteolytic event (N-terminal, C-terminal and cleavage-site spanning peptides) in a single data analysis workflow. In addition, the use of DIA effectively eliminates the missing value problem inherent to shotgun analysis.

We illustrate the power and flexibility of our next-generation positional proteomics workflow by analysis of exogenous protease activities on complex cell secretomes using as little as 20 µg total protein in each condition. Concomitant detection of the cleavage-site spanning and a neo-terminal peptide allowed determining relative degrees of substrate processing and added pivotal information for characterizing proteolysis in native proteomes. Moreover, the flexibility in experimental design enabled us to monitor activities of endogenous caspases in apoptotic monocytes with high temporal resolution. Next-generation positional proteomics will accelerate mapping of cleavage events to unravel protease dynamics in complex cellular and tissue responses.

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The Discovery of Kidney injury-related Protein Biomarkers associated with first-line Antiretroviral Treatment in South Africa

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Background: Antiretroviral therapy (ART) is made freely available to 90% of HIV positive patients in South Africa which accounts for ~10% of the global HIV/AIDS burden.The majority of patients benefit from ART, however it causes major side effects in others. This study is focussed on nephropathy related side effects. Current tests for nephropathy centre on increased urinary creatinine output. However, this manifests as a clinical marker once significant kidney damage has occurred. By identifying renal protein biomarkers patients can be monitored for these markers and thus early interventions can be made to limit further ART-related complications. This study aims to discover and validate putative protein biomarkers associated with nephropathy in HIV positive patients undergoing first-line ART.

Methods: Extracted urinary proteins from a cohort of HIV positive patients undergoing first-line ART with cases (N = 50, nephropathy) and controls (N = 50, no nephropathy), was used in this study. Quantitative proteomic workflows have been developed for urine using Norgen Urinary Protein kits to collect, store and isolate proteins. Protein expression levels are monitored by SWATH-MS using a Dionex Ultimate 3000, in microflow configuration, coupled to a TripleTOF® 6600 mass spectrometer.

Results: This urinary proteome workflow reproducibly identifies ~600 and ~900 proteins using 1D RP and 2D RP/RP, respectively. A pilot study on a subset of patients showed changes in expression of known markers of kidney damage such as β-2 Microglobulin and Retinol Binding Protein.

Conclusion: Using the developed methodology, numerous candidate markers have been identified and will be further assessed in the entire cohort. The proteome analysis data, demographic and clinical assessment data will be collated and analysed to determine whether any correlation exists between patients' proteomes and key clinical outcomes related to first-line ART.

Keywords: urinary proteome, HIV/AIDS, SWATH, 2D-RP/RP

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cIAPs as regulators of FALS-associated mutant SOD1

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The inhibitor of apoptosis protein (IAP) family is an endogenous and negative regulator of apoptosis. IAPs are characterized by the presence of the N-terminal baculovirus IAP repeat (BIR) domain. Initially, IAP proteins have been shown to inhibit apoptosis by directly preventing proteolytic cleavage of caspases. In this present study, we demonstrate that cIAPs selectively bind to mutant SOD1 proteins, and that this interaction facilitates the ubiquitination of mutant SOD1, leading to the degradation of mutant SOD1. Furthermore, we show that knock-down or pharmacologic depletion of cIAPs induces H2O2 induced cytotoxicity in mutant SOD1 expressing cells. Our findings implicate cIAPs as regulators of FALS-associated mutant SOD1 and provide an evidence that cIAPs could be therapeutic target for FALS.

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MS/MS-free protein identification in complex mixtures using multiple enzymes with complementary specificity

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Background
Protein identification without a use of tandem mass spectrometry was an appealing approach for proteome characterization. Peptide mass fingerprinting was in the beginning of this concept, however, its utility has been limited by low specificity of the peptide MS1 spectra alone and deficiencies in the mass spectrometry. Recent progress in high resolution mass spectrometry regained an interest in the MS/MS free characterization of complex protein mixtures. However, the strategy, which allows overcoming the shortage of specificity of MS1 spectra for efficient protein identification, has yet to be developed.

Methods
In this work, we developed and evaluated a workflow based on the parallel multi-enzyme digestion of the analyzed protein mixture for MS1-only protein identification. The proposed database search also employs peptide retention time prediction. The MS-only workflow was evaluated using LC-MS data for UPS1 standard and HeLa cell lines. The strategy was implemented as an open-source software based on the Pyteomics library.

Results
For the relatively simple mixtures such as the UPS1 protein standard, the proposed MS1-only approach allowed up to 90% of the UPS proteins being unambiguously identified. Up to 700 and 1200 proteins were identified at the 1% FDR level when using single and multiple enzymes, respectively for the HeLa cell line. Importantly, the sequence coverage for the identified proteins was almost ten-fold higher compared with MS/MS-based search. Another interesting feature of developed MS1-only search is its independence of the chromatographic gradient time compared with the commonly used MS/MS-based proteome analysis.

Conclusions
The utility of the proposed approach can be found in the applications when the speed of the analysis is more important than its depth, such as the clinical trials of statistically large number of samples, rapid quantitative profiling of the major components of the proteomic samples, etc.

Keywords
bioinformatics, peptide-mass fingerprinting, protein identification

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Novel capillary-flow LC-MS platform for robust proteomics profiling of cell lysates and bio-fluids

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Background
Deep, high throughput LC-MS profiling in complex matrices is a common tool to reveal changes in the proteome caused by external factors or diseases. Recently, we showed that capillary-flow LC-MS (capLC-MS) combines comparable high sensitivity of nanoLC-MS with the throughput and robustness of analytical flow LC-MS. In this work, we proposed and tested a novel capLC-MS platform with 150 µm ID EASY-Spray columns and a new ultra-high sensitive, high-resolution accurate-mass (HRAM) Orbitrap MS for deep and robust label-free quantitative proteome profiling.

Methods
The capillary-flow UltiMate 3000 RSLCnano system was configured in pre-concentration or direct injection mode and was coupled to a HRAM Orbitrap MS. Data were acquired with Xcalibur.

Results
We evaluated the capLC-MS performance in terms of sensitivity, chromatographic performance, throughput and robustness. The sensitivity of capLC-MS is 20 to 80 times higher than analytical flow LC-MS and only 2-4 times lower than nanoLC-MS under the same loading conditions. We developed capLC-MS methods for fast proteome profiling of cell lysate digest and plasma digest with 60 and 90 min total analysis time. The peak width at half maximum was < 15 sec at a flow rate of 1.2 µL/min. The number of reliably identified protein groups reached 4000 with a 60 min DDA acquisition. The high loading capacity of the capillary column allowed to load up to 5 µg of cell lysate digest onto the column without major impact on chromatographic performance. Additionally, we proved that capLC-MS can be used for long-term experiments and analysis of large sample cohorts.

Conclusions
A novel capLC-MS platform that combines capillary-flow UHPLC, 150 µm ID EASY-Spray column and HRAM Orbitrap MS is well-suited for deep and robust routine proteomic studies.

Key-words
Capillary-flow LC-MS
Proteomics
Robustness

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Linking genotype to clinical phenotype through SWATH-MS analysis of chronic lymphocytic leukaemia trial samples

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Background

Chronic Lymphocytic Leukaemia (CLL) is the most common leukaemia in the West. It is notorious for its variable clinical course and response to therapy, suggesting a role for personalised medicine. The molecular basis of CLL variability remains incompletely understood. We have embarked on a proteomic study on 350 CLL trial samples for which whole genome sequencing data will be available through the GEL 100,000 Genomes Project. Here we describe a mass spectrometric platform for screening CLL patient samples using data independent acquisition (DIA).

Methods

A CLL-specific database, generated from 16 pooled samples, was created using data dependant acquisition. The database was used to support quantification of proteins by SWATH (Sequential Windowed Acquisition of all THeoretical fragments) DIA.

Twenty CLL patient samples were prepared in replicate. Peptides were delivered into a TripleTOF 6600 mass spectrometer (SCIEX) via an Eksigent nanoLC 415 system (SCIEX). SWATH analysis was performed in triplicate, using 100 variable windows of 5 Da effective isolation width to cover a mass range of 350-1250 m/z. Variability in and between technical batches were assessed.

Results

Our CLL-specific database contains over 1.1 million spectra, containing digital information for 98,218 peptides to enable the quantification of 7416 proteins by SWATH. Technical reproducibility between SWATH runs was shown to be high, with coefficient of variation (CV) of 2.43%. Sample preparations showed batch effects. However, various batch correction algorithms were assessed and technical variabilities were successfully removed. In addition, statistical power analysis based on the results of a quadratic linear model is being used to calculate the number of patient samples required to detect differentially expressed proteins at high statistical power.

Conclusions

SWATH in combination with systems biology will enable the influence of genomic mutations on protein expression to be elucidated and improve the prediction of clinical outcome.

Keywords

SWATH, Leukaemia, Clinical proteomics.

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Unexpected covalent modification of protein histidine residues by drug metabolites in vitro and in vivo

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Background: The generation of reactive metabolites is of concern in drug development because of the risk of drug toxicities including hepatotoxicity and hypersensitivity. A large number of drugs associated with idiosyncratic drug toxicity in man undergo bioactivation and have been shown to form covalent adducts in vitro and more recently in patients. Characterization of the adduct structure and the binding sites in proteins formed by reactive metabolites will allow us to better understand the molecular basis of drug toxicity associated with bioactivation. A variety of amino acids may be targeted, in which lysine and cysteine have been the most common targets for many drug metabolites. Covalent binding to histidine has been reported rarely, probably due to the formation of unstable and reversible adducts. In this study, we report a range of unexpected histidine adducts formed by drug metabolites that were characterized by LC-MS/MS.
Methods: Human serum albumin (HSA) was used as a surrogate protein for investigating the chemical reactivity of drug metabolites in vitro. A range of drug metabolites including quinone imines (diclofenac and lapatinib), epoxide (carbamazepine), and α,β-unsaturated aldehyde (abacavir) were incubated with HSA. The protein was purified, digested, and analysed by LC-MS/MS.
Results: Quinone imines and α,β-unsaturated aldehyde formed histidine adducts with His146 and His338 in HSA via 1,4-addition. In the case of epoxide, the reaction of the 10,11-carbamazepine epoxide (a stable metabolite of carbamazepine) with histidine proceeds through an SN2 mechanism. Importantly, A same carbamazepine histidine adduct (His146 in HSA) was also identified in the plasma from patients exposed to the drug.
Conclusions: LC-MS/MS analysis revealed unexpected adducts on histidine formed by a range of drug metabolites. These adducts appeared to be stable and persisted in patient plasma, suggesting that they have the potential to trigger adverse drug reactions in patients.
Keywords: Drug metabolites, covalent binding

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OMICS based stratification of dilated cardiomyopathy patients

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Background
Dilated cardiomyopathy (DCM) is heart failure (HF) characterized by ventricle dilation and loss of contractile function. DCM patients have a poor prognosis and high incidence of cardiac transplantation. Early diagnosis and identification of patient subclasses are urgent prerequisites for the development and application of personalized therapies. Based on blood plasma protein profiling of DCM patients we aimed to (i) characterize the molecular profile associated with different disease states; (ii) find early indicators for disease progression; and (iii) define signatures for the identification of therapy responders.
Methods
We analyzed specimen of well phenotyped DCM patients of the SFB/TR19 cohort. In plasma a global protein profiling on an UPLC-ESI-LTQ-Orbitrap-Velos mass spectrometer (MS) in combination with label-free quantitation was performed and extended by an antibody based targeted analysis (Proximity Extension Assay technology) of low abundant proteins with association to cardiovascular diseases. Findings were validated by targeted MS analyses and ELISA and compared with data of patients with other HF etiologies.
Results
Plasma protein profiling indicated increased levels of acute phase response (APR) proteins in DCM patients in comparison to subjects with normal ejection fraction. Moreover, significantly lower amounts of serum paraoxonase 1 were detected , obviously being specific for cardiomyopathies but not found in patients with diastolic dysfunction. Spontaneous improvement of cardiac function in DCM patients under standard medication was indicated by lower levels of proteins involved in defence response. Mortality of patients was associated with higher levels of proteins known to be related to risk for cardiovascular events. Addressing response to immunoadsorption therapy, higher levels of APR proteins were found to be associated with improved contractile function in DCM patients.
Conclusions
Plasma protein profiling approaches are helpful identifying circulating molecular markers supporting stratification of HF patients with different etiopathologies and selection of optimal individual therapy.
Keywords
DCM; plasma profiling, OLINK, biomarker

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Discovering proteome alterations in PARK2-mutated Parkinson's disease patients.

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Background
Mitophagy impairment is one of the most important molecular hallmarks of Parkinson's disease (PD). One of the recently proposed mechanisms for mitophagy in mammalian cells is the Pink1-Parkin-mediated mitophagy pathway. Noticeably, mutations in the genes that encode for these two proteins, respectively PARK6 and PARK2, are associated with autosomal recessive parkinsonism. In particular, Parkin protein is an E3 ubiquitin ligase involved in the selective degradation of damaged mitochondria. For these reasons, mutations in the PARK2 gene, which cause the loss of function of Parkin, are responsible for the accumulation of damaged mitochondria that may generate higher levels of ROS, a lower ATP production and apoptosis activation.
Methods
Mitochondria were isolated from skin fibroblasts cell lines of 3 control subjects and 3 PARK2-mutated patients using a commercial kit based on surfactants. Mitochondrial protein extracts were then analyzed by quantitative shotgun proteomics (Synapt G2, Waters). An immunofluorescence staining of ATP synthase was also performed in order to analyze the mitochondrial network shape.
Results
Shotgun proteomics analysis revealed a large number of mitochondrial proteins that quantitatively or qualitatively change in PARK2-mutated patients, e.g. NADH dehydrogenase [ubiquinone] flavoprotein 2 and Lon protease homolog. Surprisingly, no difference in mitochondrial network organization was detected between the two groups.
Conclusions
Our proteomics data suggest that the loss of function of the Parkin protein causes respiratory chain dysfunctions and alterations of the mitochondrial quality control system. Moreover, PARK2 mutations do not affect the mitochondrial network shape. Possibly, this observation could reflect the improper disposal of dysfunctional mitochondria that remain in the network as a consequence of the mitophagy impairment.
Keywords
Parkinson's disease, mitochondria, proteome.

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Comprehensive proteomic description of ontogenic changes in haematopoietic stem and progenitor cells

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BACKGROUND
Increasing evidence indicates that blood stem cells in foetus and adult possess distinct molecular landscapes that regulate cell fate and change their susceptibility to initiation and progression of blood cell cancers. The proteomic programs that drive this difference in foetal and adult blood cell development remain elusive. In this study, we have utilized a mass spectrometry-based quantitative proteomics approach to comprehensively describe and compare the proteome of foetal and adult blood stem and progenitor cells.

METHODS
Mouse foetal liver and adult bone marrow haematopoietic stem and progenitor cells (HSPCs; Lin-Sca1+cKit+) were FACS-sorted in three replicates and subjected to quantitative proteomics workflow including dimethyl labelling, peptide pre-fractionation and LC-MS/MS analysis on QExactive Plus.

RESULTS
Close to 7000 proteins were identified, allowing us to comprehensively describe the proteomic differences between these cells. 454 proteins showed differential expression, indicative of the divergent nature of foetal and adult haematopoiesis. While the proteome of foetal HSPCs is fairly simple, with main features being cell cycle and cell proliferation, their adult counterpart has a more advanced proteome, including an arsenal of proteins important for viral and bacterial defense, as well as protection against ROS-induced protein oxidation. Our further analyses of Type I interferon signalling showed that foetal HSPCs are sensitive to Interferon alpha (IFNa) in vitro, although their exposure and response to IFNa in their native environment is limited.

CONCLUSIONS
Our results provide new and important insights into the molecular landscape of foetal and adult haematopoiesis that advance our understanding of normal and malignant haematopoiesis during foetal and adult life.

KEYWORDS
Haematopoiesis, Cellular proteomics

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In vitro characterisation of terbinafine protein adducts.

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Background: Idiosyncratic drug induced liver injury (iDILI) is a severe adverse reaction which manifests in a small percentage of drug recipients. Several associations have been found between iDILI drugs and allelic variants of human leukocyte antigen (HLA). Therefore, these drugs may be activating the immune system like drug hypersensitivity reactions. Patient studies in co-amoxiclav and flucloxacillin induced liver injury have used LC/MS methods to characterise drug-modified proteins in patient serum which could activate an immune response via a hapten mechanism. Subsequent studies have also demonstrated T-cell activation in these drugs. The role of the immune system in terbinafine induced liver injury and its associated allele, HLA-A*33:01, has not yet been studied. Terbinafine can be metabolised to form a stable reactive metabolite, an allylic aldehyde, 7,7-dimethylhept-2-ene-4-ynal (TBF-A). Like β-lactam antibiotics, it may form drug-protein haptens to activate an immune response. This study defines the chemistry of terbinafine-protein adducts and begins to investigate their potential to activate an immune response.

Methods: Direct incubations with TBF-A were performed with small molecule trapping agents and whole proteins. Co-incubations were also performed with human liver microsomes and the parent compound to generate the TBF-A metabolite in situ along with trapping agents. The resulting adducts were characterised using mass spectrometry.

Results: TBF-A binds n-acetyl cysteine and glutathione but not n-acetyl lysine. Additionally, TBF-A modified cysteine and histidine adducts were found upon incubations with glutathione s-transferase pi and human serum albumin. Synthetic cysteine containing peptides were generated and shown to bind TBF-A. Finally, human liver microsome trapping experiments demonstrated the presence of TBF-A adducts upon incubation with the parent drug molecule.

Conclusion: TBF-A binds small molecules, proteins and synthetic peptides. T-cell assays are being conducted to investigate the role of these TBF-A adducts in activating an immune response.

Keywords: terbinafine; adduct; DILI; liver microsome; drug hypersensitivity

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Automated proteolytic digestion for high precision peptide mapping and targeted bioanalysis

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Background
The bio-pharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Detailed bioanalysis of the new biological entity plays a fundamental part during discovery and development. To ensure safety and efficacy structural characterization is crucial at any step of the biologics product lifecycle. Peptide mapping analysis can be used to address several critical quality attributes (CQA) and is indispensable for the characterization of any biotherapeutic protein.

Proteolytic digestion is a shared bottle neck during sample preparation for both targeted bioanalysis and peptide mapping analysis. Although a widely accepted technique, in-solution trypsin digestion protocols are labor intensive and prone to manual errors. These errors affect the quality of the analytical data in both bioanalysis and peptide mapping experiments, thus compromising the ability to reproducibly characterize a protein to the required standard.

Method
This work details on an automated digestion workflow that mitigates against manual errors during sample preparation. The Thermo Scientific™ SMART Digest™ heat-stable trypsin allows to vastly accelerate the digestion process and has been shown to facilitate reliable peptide maps and increased sensitivities in targeted protein quantification assays. Here a magnetic bead support is applied to immobilize the SMART Digest trypsin and is used in combination with the Thermo Scientific™ KingFisher™ Duo magnetic bead purification system to automate the digestion workflow. Cytochrome C, recombinant Somatotropin and Infliximab drug product were chosen to investigate the applicability and reproducibility of this automated digestion protocol and the subsequent peptide mapping analysis.

Results and Conclusion
The KingFisher purification system enables robotic handling and easy automation of the magnetic SMART digest resin and produces high-resolution peptide maps with superior performance and reproducibility.

Keywords:
Automation, Peptide Mapping, Bioanalysis, Biotherapeutic

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Proteomics analysis and lysine acetylation stoichiometry in cancer cells.

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Protein lysine acetylation is involved in several processes that maintain the proper functioning of cells, including transcriptional regulation and metabolic functions. Therefore, acetylation of lysine residues has emerged as a crucial PTM for a wide range of cellular processes and is involved in aging and the development of several diseases, including cancer. Recent studies revealed that lysine acetylation sites mainly exhibit low stoichiometry.

Here we explored three sample preparation methods, using detergents for the chemical acetylation reaction in combination with a stable and efficient alkylating reagent, to analyze the stoichiometry of acetylation in three human cell lines. We identified and determined the acetylation occupancy in approximately 1 500 proteins from each cell line.
The stoichiometric analysis, in combination with quantitative proteomics, allowed us to gain a better understanding of the role of this PTM in different cells. We found that higher abundance of the deacetylase SIRT1 correlates with less acetylation occupancy and abundance of ribosomes, as well as proteins involved in ribosome biogenesis, rRNA processing, among others.

We confirmed, through the inhibition of SIRT1 with6-Chloro-2,3,4,9-tetrahydro-1 H-Carbazole-1-carboxamide (EX-527), a potent and specific inhibitor of the deacetylase enzyme SIRT-1, followed by quantitative proteomics and acetylation stoichiometry analyzes, the negative role of this deacetylase on transcription and translation pathways. In addition, we found that SIRT1 positively regulates several metabolic pathways. Park of this work was supported by a DGAPA-PAPIIT grant IN213216 and CONACYT grant 220790.

Keywords: Acetylation, stoichiometry, cancer, SIRT1.

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Definition of the intracellular flucloxacillin-haptenated proteins that may have involvement in iDILI

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Background
Adverse drug reactions (ADRs) represent a major clinical concern and impediment to the drug development process, yet their precise molecular mechanisms are not well understood. The hapten hypothesis proposes that covalent modification of biological macromolecules with drug may drive immune-mediated ADRs. Flucloxacillin, a broad range beta-lactam antibiotic, may induce idiosyncratic drug-induced liver injury (iDILI) which cannot be predicted based upon the drug pharmacokinetics. While HLA-B*57:01 has been linked to flucloxacillin iDILI, suggesting immune involvement, only 8.5 in 100,000 people develop ADRs, much lower than the frequency of the allele. Currently, little is known about the proteins that are haptenated by flucloxacillin, and whether the modification of intracellular proteins leads to the presentation of functional antigenic drug-modified peptides.

Methods
Flucloxacillin was conjugated to ovalbumin in vitro to act as an immunogen for antibody production and was fully characterised by LC-MS. The antibody was used as a precise tool for pinpointing intracellular haptenated proteins that were subsequently identified by state-of-the-art LC-MS. This approach was used to study the uptake, distribution and protein binding of flucloxacillin in immortalised and primary cells from liver and the immune system.

Results
A high titre, flucloxacillin-specific polyclonal antibody was generated that cross-reacted with the oxacillins, but not with other beta-lactams. The immunogenic region of flucloxacillin was hypothesised and molecular docking studies performed, providing perspective on how flucloxacillin hapten may interact with the immune system. Protein targets of covalent modification were identified, and their potential contribution to the development of iDILI will be explored.

Conclusions
The hapten hypothesis was proposed in 1945 (Landsteiner), but no unequivocal evidence for its role in drug hypersensitivity reactions has been acquired. The combination of highly sensitive hapten-specific antibodies and state-of-the-art MS provides a platform for linking chemistry with biology and for dissecting the mechanisms of iDILI.

Keywords
Flucloxacillin, Liver Injury, Hypersensitivity.

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Peptidomics: an innovative approach to Study the “Auto-digestion” hypothesis in Septic Shock Patients

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Background: Recently, Schmid-Schöbein and Chang hypothesized that the “auto-digestion” phenomenon is highly involved in Multi Organs Failure associated to Shock. In this study, we applied a peptidomics approach in order to evaluate the level of proteolysis in blood plasma samples from 18 Septic Shock (SS) patients taken at three time points: 24h (T1), 48h (T2) and 7 days (T3) after ICU entry, 7 control patients (sepsis at T1) and 9 healthy donors (NTOT = 70). SS patients were divided into three groups (NR_A, NR_D, R_A) according to the response to vasopressors therapy (Non responders (NR), defined as having SOFA score T2 > 8 and ΔSOFAT1-T2 < 5) and to 28-day survival (A = Alive; D = Dead).
Methods: Samples obtained under informed consent from patients were processed as follow: 50 µL were ultrafiltered by 10KDa filter and cleaned up using Oasis HLB PRiME µElution plates and SCX Top Tip chromatography. Samples were analyzed by LC-MS/MS and quantified (label-free) in Progenesis QI software.
Results: The highest proteolysis level (mean of proteins intensity) was observed at T3 for the NR_D group. This could be due to a higher level of incessant proteolysis in this group. On the other hand, the “Alive” groups showed decreased mean intensities towards T3 with values comparable to the mean intensity observed in the healthy group, which presented the lowest value. ANOVA analysis showed significant differences when samples T1 and T2 of NR_A and R_A were compared. 16 differentially proteolyzed proteins, including higher level of Histone 1.4 proteolysis in NR and higher level of α-2-antiplasmin in R, were identified.
Conclusion: As far as we know, these results support the “auto-digestion” hypothesis in shocked human patients. Proteolysis seems to correlate with survival and some proteolyzed proteins could be predictors of response to therapy.
Keywords: Peptidomics, Septic shock, Proteolysis

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Early warning signals of aging in the hematopoietic stem cell niche.

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Background
The limitation of life and aging has always been in the interest of mankind. As certain common diseases are predominantly arising when we are aged, the socio-cultural aspect of aging and the medical interest on the process of getting older will touch almost everyone in our society.

Methods
We analyzed cells originating from human bone marrow of 59 donors in the age between 20 to 60 years. Cells were lysed and the proteins are digested with Lys-C followed by trypsin. The resulting peptides were labeled with the tandem mass tag (TMT). Samples from five donors and one internal standard were mixed together and separated by reversed phase chromatography at basic pH. The fractions were analyzed via a nanoflow reversed phase liquid chromatography system directly coupled to a high resolution mass spectrometer. The identification of peptides is conducted with standard software and the results were further processed with an in house build pipeline including filtering, quantification, and quality assessment.

Results
In our current study, we are interested in the effect of aging on the hematopoietic system in humans. Here, we characterize the normal perturbations of the hematopoietic system during aging. Several proteins in different cell populations originating from the human bone marrow change in abundance during our lifespan. This allowed unravelling the bio-molecular processes in hematopoiesis that are responsible to known phenotypic alterations upon aging in humans.

Conclusions
During our lifespan the hematopoietic system is subject to molecular changes. These changes in the proteome are not random, but many of the proteins whose abundances change during aging are described to interact with each other. Here, we describe some of these interactions that give rise to the alterations upon aging in the human hematopoietic system.

Keywords
Hematopoietic stem cells, aging, human, bone marrow

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The ProteomeXchange Consortium: 2017 update

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Background. The overall aim of the ProteomeXchange (PX) Consortium (http://www.proteomexchange.org) is to enable proteomics data sharing by providing a common framework and infrastructure for the cooperation of mass spectrometry proteomics resources (PMID: 24727771). PX defines and implements consistent, harmonised, user-friendly data deposition and dissemination procedures among its members (PRIDE, PeptideAtlas, MassIVE and since 2016, jPOST). Thanks to the success of PX, public availability of datasets in the field has become the norm, and as a result, reuse of public proteomics data is increasing steadily.
Methods. Currently, PRIDE (EMBL-EBI, Cambridge, UK), MassIVE (University of California San Diego, USA), and jPOST (various Institutions, Japan), although focused in tandem MS/MS experiments, can store data coming from any type of proteomics approach, while PeptideAtlas (Institute for Systems Biology, Seattle, USA) provides a repository for Selected Reaction Monitoring experiments called PASSEL. The PX members actively recommend the use of the open data standards developed by the Proteomics Standards Initiative, including mzML, mzIdentML and mzTab.
Results. The implementation of PX has resulted in a fast increase of publicly available proteomics datasets – by May 2017 more than 6,000 datasets had been submitted to any of the four PX partners, totaling over 320 TBs and thousands of MS runs. Apart from the individual resources, the main common access point is ProteomeCentral (http://proteomecentral.proteomexchange.org), which provides the ability to search datasets in all participating PX resources. Additionally, PX datasets are also available via the recently developed Omics Discovery Index (OmicsDI, http://www.omicsdi.org). It is possible for anyone to follow the announcements of new public datasets, via e-mail, RSS or Twitter (@proteomexchange).
Conclusions. With strong support from funding agencies and scientific journals, PX is contributing to changing the "culture" in the field, by promoting and enabling efficient sharing of proteomics data in the public domain.
Keywords. computational proteomics, databases, open data.

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A quantitative comparative proteomic approach to investigate Hülle cells from the filamentous fungus Aspergillus nidulans

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A. nidulans sexual and asexual differentiation is linked to significant differences in formation of secondary metabolites and the formation of various distinct cell types in the different developmental programs. A comparative approach to investigate differences in the proteomes of fungal cells of different developmental programs was performed. 870 proteins could be identified in vegetative mycelium, 1046 proteins were identified in hyphae where sexual development was induced (dark conditions) and 877 proteins in hyphae where asexual development was induced (illumination). Hülle cells are unique cell types of Aspergilli and are linked to the sexual program. These globose thick-walled cells presumably support the formation of cleistothecia which are closed sexual fruiting bodies. A protocol concerning the enrichment of Hülle cells was established where 401 proteins could be identified. A comparative analysis of all proteins identified in the different mycelia mentioned above was performed. 299 proteins were specifically identified in vegetative mycelium and these proteins did not overlap to others identified in this study. During sexual development 203 proteins were specifically identified. In asexual mycelium 118 proteins were uniquely identified. In this study 6 proteins were unambiguously identified in the enriched Hülle cells that could not be identified in other different mycelia types. Our results were compared to vegetative mycelia lacking the methyltransferase LaeA that causes drastic reduction in the secondary metabolism and in the formation of Hülle cells. To study proteins that are under the regulation of LaeA with high and low amounts of Hülle cells laeA was deleted in a lysine auxotrophic parental strain. These comparisons allow to identify and quantify proteins under the regulation of LaeA in a mycelium with high and low amounts of Hülle cells using stable isotope labeling by amino acids in cell culture (SILAC).

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Tissue proteomics unravel EIF3D as a potential candidate associated with bladder cancer invasion

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Background
Tissue proteomics may provide a wealth of information on the mechanisms of bladder cancer (BC) initiation and progression as well as on the identification of novel druggable targets. To this end, this study focuses on the global characterization of the tissue proteomic changes underlying BC invasion.

Methods
LC-MS/MS analysis of tissue specimens from patients with non-muscle invasive (NMIBC, stage pTa, n=5) and muscle invasive BC (MIBC, stage pT2+, n=6) was conducted. To increase the validity, the raw MS data were processed and evaluated by two independent software packages and also confirmed by IHC on an independent tissue set. In silico pathway and protein interaction analysis predicted candidates whose impact was investigated in vitro in the metastatic properties of T24M cells, and in vivo in mouse xenografts.

Results
Comparative tissue proteomics analysis identified 144 differentially expressed proteins at statistically significant levels between NMIBC and MIBC samples. These included proteins that were previously reported in the context of BC (e.g. ACTN4, CTSE, CDH13) as well as novel findings (e.g. PGRMC1, FUCA1, BROX and PSMD12). The latter were confirmed by IHC on an independent tissue set (n=15). Pathway and interactome analysis predicted strong activation of pathways associated with protein synthesis (e.g. eIF2 and mTOR signaling) in MIBC. EIF3D, a component of the translational machinery, was found up-regulated in MIBC. EIF3D knockdown in the metastatic T24M bladder cancer cells reduced cell proliferation, migration and colony forming ability in vitro and decreased tumor growth in vivo. By contrast, knocking down RHEB which is upstream of mTORC1, recapitulated the effects of EIF3D knockdown in vitro, but not in vivo.

Conclusions
Collectively, this study represents a comprehensive proteomics analysis of NMIBC and MIBC. The results highlight EIF3D as a potential therapeutic target in BC.

Keywords
Tissue proteomics, bladder cancer, translation, EIF3D

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Strategies for Identification of Organ-Specific Biomarkers of Exposure to Ionizing Radiation

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Background;
Humans and other mammals exhibit complex responses involving multiple organs following exposure to sub-lethal/lethal doses of ionizing radiation (IR) dependent upon dose and extent of exposure. IR-damage to bone marrow, gastro-intestinal tract, lung and other organs can all lead to lethality in the weeks and months following exposure. Measurement of time-dependent changes in protein signatures in plasma following radiation exposure offer the potential to predict organ-specific damage to steer intervention and assess efficacy of mitigation.
Methods;
Discovery proteomics is being used to measure changes in protein expression in organ systems (bone marrow, lung and gut) in mice at 1-, 2-, 4-, 8- and 30-days after exposure to 6 Gy total body IR, a dose known to cause hematopoietic syndrome. Proteins showing significant change are sorted into pathways and panels of potential predictors are subsequently measured in plasma using targeted assays. Given the known role of the transcription factor Nrf2 in the cellular response to radiation we focused one set of experiments on the proteins most prominently modulated by Nrf2 and the anti-oxidant response element (ARE) promotor that it targets.
Results;
Different pathways were classified into different categories based upon their changes in expression over the time course following IR exposure. For a mass triage scenario we are particularly interested in pathways that show elevation over the 4- to 8-day period. The Nrf2 family falls into a class that shows elevation at day 1 with a return toward baseline in the following days.
While most of the Nrf2-modulated proteins, such as SOD1 and catalase, are detectable in extracts from bone marrow, detection in plasma is much more challenging, but will be required for translation into a biomarker assay.
Conclusions;
IR stress causes widespread, dynamic proteome remodeling.
5 Keywords
ionizing radiation, pathways, ARE promotor, temporal response, mitigators,

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lcmsWorld: A high-performance 3D software visualisation tool for LC/MS data

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Background
Liquid Chromatography (LC)-Mass Spectrometry (MS) is widely used to ascertain the constitution of biological samples, often for identification and quantification of metabolites, lipids, or peptides (as proxy markers for the parent proteins). In most workflows, analytical software is used for correctly identifying data within the LC/MS spectra for detection and quantification of these substances. Problems with analysis, such as misidentifications or unreliable quantification, can happen for many reasons, including peptides or metabolites of similar masses eluting together through the LC stage, which software can struggle to differentiate. An experienced eye may better understand the underlying cause of such complex data regions but there is currently limited software support to facilitate this, hence we have designed new software for this purpose.
Methods
Our new software – lcmsWorld – aims to provide an environment in which the scientist can quickly and easily browse and view LC/MS data in three dimensions. The software uses the power of modern graphics chips alongside carefully managed access to the data to enable fast performance. It is written using C++ for efficient computation and uses the OpenGL standard, which focuses on High Visual Quality and Performance, and supports almost all 3D graphics chips across different computing platforms.
Results
lcmsWorld displays LC/MS data as a three-dimensional data 'landscape' - going from a complete dataset overview, which the user can navigate around and 'zoom-in', to view the individual data points of the LC-MS spectra as 'peaks' in the landscape. This data may be loaded in a variety of manufacturers' file formats and the community standard mzML format.
Conclusions
lcmsWorld can help scientists to get a better understanding of the data that was actually created by the equipment, and can help them understand, prevent and correct any errors that may occur in downstream analysis.
Keywords
LC MS Software visualisation

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How can we effectively detect biomarker candidates by mass spectrometry?

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Background: It is challenging to detect disease-related(DR) proteins among many proteins in human samples, and particularly difficult in serum or plasma, largely because we cannot easily determine if a change in protein expression is due to disease(s), individual differences, or experimental error by mass spectrometric analysis. Therefore, innovative methods are required in order to efficiently detect DR-proteins. We used three sample preparation methods to more easily and efficiently detect DR-proteins that could be used as diagnostic markers.
Methods: 1) We detected DR-proteins secreted from cultured cells (secretome analysis), and investigated whether they could be used as diagnostic markers in serum/plasma. 2) After depleting high-abundance proteins of serum/plasma, we detected DR-proteins by comparing the serum/plasma proteins of the "same person" in the healthy and diseased states. 3) We enriched particular protein species, such as phosphoproteins, and detected DR-proteins among the enriched proteins.
Results: First, we detected the ovarian clear cell carcinoma(CC)-related protein TFPI2 by secretome analysis. TFPI2 was up-regulated in the serum of CC patients. Studies using a large number of clinical specimens suggested that TFPI2 could be used as a diagnostic marker. Second, we found Kawasaki disease–related proteins using serum from the same subject collected during the acute and recovery periods. Clinical trials using a large number of specimens revealed that expression of these proteins is correlated with the symptoms of Kawasaki disease, suggesting that these proteins could be used as a diagnostic marker. Third, enrichment of DR-proteins, such as DR-phosphoproteins, was also an effective means to identify diagnostic markers by mass spectrometry. Using a phosphoprotein enrichment technique, we identified phosphoproteins whose phosphorylation changed drastically during the epithelial–mesenchymal transition, and showed that these proteins could predict the prognosis of lung adenocarcinoma.
Conclusion: The sample preparation methods used here were essential to efficiently detect DR-proteins as diagnostic markers.

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Next-generation blood biomarkers for acute liver injury:
in silico discovery and proteomics evaluation

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Acute liver injury (ALI) is a severe disorder resulting from excessive hepatocyte cell death and frequently caused by acetaminophen intoxication. ALI can rapidly progress to acute liver failure (ALF), a systemic and life-threatening condition. The paucity of blood biomarkers of ALI progression makes its clinical management difficult. The goal of this study was to explore the huge amount of information generated by large-scale biology to discover new mechanistic biomarkers for ALI.

Bioinformatics databases were explored to select proteins with a liver-predominant expression and a high detectability in the blood. Using an innovative and efficient proteomics pipeline based on targeted mass spectrometry (PSAQ-SRM), six proteins were assessed as potential biomarkers of hepatocyte cell death in serum samples from patients with ALI or ALF of different aetiologies.

In patients with acetaminophen-induced ALI/ALF, the serum concentration of alcohol dehydrogenase 1 (ADH1), alcohol dehydrogenase 4 (ADH4) and betaine-homocysteine S-methyltransferase (BHMT) markedly increased during the acute phase of the disease and dropped to undetectable levels during the recovery period. This time-progression reflected liver cytolysis, very specifically and accurately. The combined interpretation of ADH1 and INR kinetic patterns also appears useful for prognosis evaluation. In patients with non-acetaminophen-induced ALI/ALF, variable but significant increases in serum ADH1, ADH4 and BHMT concentrations were also observed, except for the autoimmune aetiology.

ADH1, ADH4 and BHMT emerged as novel candidate biomarkers to detect drug-induced liver injury and evaluate the severity and progression of ALI.

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Multiplexed Activity Profiling of Cellular Signaling Pathways by Data-dependent and Targeted LCMS

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Background
The challenge for biomedical research in the post-proteomic era is to develop new generations of tools to analyze complex biological systems and that simultaneously interrogate whole networks of signaling pathways. Understanding biological behavior is multifactorial, and necessitates analytical tools that are amenable to high degrees of multiplexing and rigorous quantitation for accurate measurement of hundreds of cellular proteins and post-translational modifications (PTMs) simultaneously within a biological system.

Methods
We have developed an enrichment reagent combining target-specific antibodies representing key signaling nodes from multiple pathways, including cell cycle control, TCR and BCR signaling, MAPK signaling, JAK/STAT signaling, DNA damage checkpoint, AKT/PI3K signaling, and many others. In contrast to Cell Signaling Technology's PTMScan reagents, in which a single antibody is used to immunoaffinity purify many peptides containing a PTM, here we utilize a mixture of protein- and phosphorylation site-specific antibodies selected to provide broad coverage of signaling pathway activity.
To profile pathway activity, proteins are proteolytically digested, and enrichment is performed at the peptide level. Enriched peptides and phosphopeptides are analyzed through data-dependent LC-MS/MS for total pathway profiling. Alternatively, key nodes may be quantified through targeted MS analysis.

Results
We have used the multipathway-profiling reagent to enrich and identify peptides from multiple human cell lines and mouse tissues. Using data-dependent LC-MS/MS, thousands of peptides are identified representing hundreds of sites of phosphorylation, enabling measurement of activity of dozens of signaling pathways.
This enrichment reagent has also been utilized for peptide purification upstream of targeted LCMS analysis, quantifying dozens of peptides with attomole sensitivity.

Conclusions
Multiplexed pathway activity profiling through sample enrichment with peptide-specific antibodies enables identification of pathways activated or inhibited by drug or ligand treatments as well as possible unpredicted off-target effects. This strategy can be applied to any protein sample type, including cell lines, tissue samples, and clinical biopsies.

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Intelligent solution for mass spectrometry-based proteomics

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Background
Mass spectrometry (MS) is the main technology used in proteomics approaches. However, most computational search algorithms detect peptides by scoring the degree of similarity between in silico derived and experimental peptide spectra, and thus can only identify peptides that are present in the proteomic database. If the polypeptide sequence is not present in the data-base used for searching, even if the peptide is present in the sample, it will fail to be detected. In this reason, on average, 75% of spectra analyzed in an MS experiment remain unidentified. In recent years, post-translational modifications (PTMs), single amino acid polymorphisms (SAAPs) and splice variant isoforms (SVIs) have been shown to be source of cell-specific proteomic variation in humans.

Methods
We propose to develop a novel software yLabel, for intelligent labeling targeted protein/peptide with PTMs,SAAPs and SVIs to shed light on these unidentified spectra. yLabel supporting potential biology variation mining from serval public databases involves dbSNP(https://www.ncbi.nlm.nih.gov/projects/SNP/), sysPTM（http://lifecenter.sgst.cn/SysPTM/), and EURASNET(http://www.eurasnet.info/tools/asdatabases). Using global similarity scoring methods and multiple complementary analysis approaches, we were able to identify these unusual spectra with the labeling peaks.

Results
By labeling the conformed the variated proteins/peptides in database, yLabel was verified to have a high precision of labeling the mark peaks. A number of potential biology variations were also identified by the tool.

Conclusions
yLabel encourages further intelligent translating of life code in proteomics.

Keywords
Unidentified spectrum, intelligent labeling, biology variation, Computational Proteomics

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UPS2 CALIBRATION SET FOR BIOLOGICAL MATRIX EFFECT STUDY

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Background

Targeted (SRM) and panoramic (Shotgun) mass spectrometric approaches are widespread in the proteomics. Complexity of lowcopied and ultralow-copied detection increases due to Chemical noise from the biological matrix molecules. Besides different search engines give variable results. We studied biological matrix effects on the protein identification results using model calibration UPS2 set (Sigma Aldrich).

Methods

In this work we used the results of SRM and Shotgun LC-MS/MS proteomic analysis of the pure UPS2 calibration set solution, UPS2 in E.Coli extract and UPS2 in human blood plasma. UPS2 set is a mixture of 48 individual human source or human sequence recombinant proteins with a dynamic range of 5 orders of magnitude, ranging from 50 pmoles to 500 amoles.
For shotgun analysis identification results of Mascot, Search GUI and MSFRAGGER were compared.
Using SRM data we estimated biological matrix effect on the qualitative and quantitave proteomic analysis results.

Results

Shotgun data analysis using different search engines and databases revealed from 11 to 33 identified UPS2 proteins. No chemical or biological modifications were identified. SRM data analysis demonstrated that biological matrix complexity has a non-linear effect on the protein quantity. Thus, for different proteins in the same sample quantitative content changed at 11-24% in E.Coli extract and in 20-47% in human blood plasma.

Conclusions

Integration of targeted and panoramic mass-spectromic approaches, different search algorithms results, information about the biological sample features can increase the understanding of the human proteome.
Keywords: Algorithms, Databases, Tandem Mass Spectrometry, Shotgun, SRM, Proteomics, biological matrix

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Exploratory affinity assays for personalized plasma profiling

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Background - Affinity assays offer versatile approaches to analyze larger numbers of samples, such as from well-characterized patient biobanks hosting clinical and omics data. Analytical advances are driven by both new technologies and growing libraries of binding reagents, however increased knowledge about the performance of an affinity reagent in a given assay and sample context is needed. There are several examples of how current affinity efforts have profiled plasma across different diseases, but strategies need to go beyond discovery and into using validated assays.

Methods - We applied different multiplexed affinity assays for discovery and validation in body fluids. Studies are designed with randomized samples to reduce unwanted bias, considering details about both the specimen and subjects. Multidimensional data processing is then applied prior to statistical analysis. Combinations of using paired antibodies, epitope mapping, as we all as immuno-capture mass spectrometry (IC-MS) confirm the target and direct the development of sandwich assays for target validation.

Results - We performed several systematic explorations of proteins profiles using highly multiplexed assays in 1,000s of plasma samples from longitudinal, clinical or case-control studies in health and disease. While discovery approaches are often fast and efficient, they demand complex validation schemes. Hence, we established workflows including IC-MS with plasma that enabled us to validate antibodies prior sandwich assay development and to describe protein complexes in plasma. We further observed that careful analysis of available clinical and sampling data is essential and found use in other omics as a guide towards successful target validation.

Conclusions - Once validated and applied in appropriately designed studies, affinity assays can contribute with candidates for personalized plasma protein profiling. Examples from projects conducted within wellness and major chronic diseases will be given to illustrate the opportunities and challenges faced while translating discoveries into validated assays.

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Molecular Proteomic Characterization of Greek Single-Breed Dairy Products

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Background/Aim: A new class of experimental bio-functional foods is currently emerging, that may render Greek products globally identifiable, re-establishing their uniqueness by guaranteeing their designated origin, whilst offering consumers health benefits. Single-breed dairy products (SBDPs) are experimental products, characterized by the fact that their production is based solely on use of milk from a single phylogenetically-, molecularly- and genetically-identifiable Greek breed of sheep of goats. The special characteristic of these products is that they definitely, and to the fullest extent, reflect/designate the exclusive features of each animal breed they originate from. Materials and Methods: In the present proposal milk from animals of indigenous Greek breeds of goats and sheep, namely Capra prisca goats, Boutsko, Kalarritiko and Karagouniko sheep, which constitute the larger part of the national flock, as well as their relevant cheese products (white cheese, feta cheese, gruyere cheese etc.) were in-depth analyzed by nanoLC-MS/MS proteomic approaches, after thorough sample preparation for peptide extraction. Results: A mean of n=500 protein groups were identified in cheese products of each animal species, reporting for the first time the proteome dataset of Greek SBDPs. High-value molecules were identified that will help towards ameliorating consumers' well-being. Conclusion: Given their high nutritional value, this novel class of products will most definitely play important part of the of the Greek dairy product armamentarium, presenting opportunities for both dairy industries and the academic community.

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Deployment of DOSCAT technology to quantify proteins involved in paediatric pneumococcal meningitis pathogenesis

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BACKGROUND

Targeted absolute quantification of proteins in a rapid, accurate and multiplexed manner is a key aspect of proteomics. Selected reaction monitoring mass spectrometry (SRM-MS) is the gold standard technique; however, it can be limited by sensitivity and development time. Therefore, assays based on immunochemical approaches like western blotting (WB) are more routinely used. We have previously shown that DOuble Standard conCATemers (DOSCATs) can merge both techniques, increasing quantitative confidence. Here, DOSCATs are deployed to quantify six proteins involved in acute bacterial meningitis caused by Streptococcus pneumoniae, illustrating the principle and utility of employing DOSCATs in clinical proteomics.

METHODS

The DOSCAT gene was synthesised de novo to concatenate tryptic peptides and antibody epitopes for six target proteins; cathelicidin, ceruloplasmin, cystatin C, myeloperoxidase, S100A8 and S100A9. The gene was expressed in E.coli and purified via a His-tag. Cerebrospinal fluid (CSF) from patients diagnosed with Streptococcus pneumoniae infection (n=20) and without infection (n=20) was analysed using DOSCAT as a standard in SRM-MS (Xevo TQS, Waters, UK) and automated WB (Wes, Protein Simple, USA).

RESULTS

All target proteins were quantified in infected CSF using SRM-MS, but only four proteins by WB (due to poor performance of antibodies). Myeloperoxidase and cathelicidin could not be quantified in control samples due to their low intrinsic abundance. Relative quantification between case and control CSF was in excellent agreement between SRM-MS and WB, as well as with existing label free proteomics and WB (calibrated by individual recombinant protein standards) datasets. Moreover, there was a very good correlation of absolute quantification values between SRM-MS and WB.

CONCLUSIONS

DOSCATs can be used to accurately quantify proteins in clinical samples, with quantification values comparable across orthogonal techniques. This demonstrates the value of DOSCAT technology through increased target protein coverage, improved data reliability, and added throughput that comes with automated WB analysis.

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Determining network topology, distance restraints and activation markers from endogenous protein complexes

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Background
Protein complexes represent the functional modules of the cell. Studying their assembly and structure in a physiological manner is fundamental to understand cellular processes and their dynamics. Complex interactions have been studied by affinity purification mass spectrometry (AP-MS) that provides bait interactors but requires multiple experiments to attribute high interaction confidence. Conversely, cross-linking mass spectrometry (XL-MS) can pinpoint protein neighborhood and structural restraints between single amino-acid residues but requires large sample amounts and high purity. We combined the ease and throughput of AP-MS with the topological and structural information of XL-MS.

Methods
We developed qAXL-MS, combining affinity purification from tagged human cell lines with on-beads cross-linking. We enriched and identified cross-linked peptides and used the software xTract to measure the relative abundance of individual cross-linked sites between biological conditions.

Results
First, we applied qAXL-MS to the osmotic stress response in HEK293 cells. We purified signaling kinase complexes and measured the differential abundance of cross-links; we found a set of activation markers that correlated and can therefore predict the active or inactive state of the kinase upon cell stimulation. Comparing cross-links between different proteins, we devised scoring functions to determine likely contaminants, the network connectivity and the complex composition before and after the osmotic stress. We then studied the conformational changes of the human TriC chaperonin. We could define a set of distance restraints that identified open and closed states and their interactions with the chaperonin substrate as previously described only in large-scale purifications of mammalian TriC complexes.

Conclusions
We developed a quantitative method to define, for a given protein complex, interaction topologies, physical interactions, distance restraints and structural activation markers from different cellular states with as little as tens of million human cells.

Keywords
Protein-protein interactions, structural proteomics, affinity purification, cross-linking, quantitative mass spectrometry.

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Novel plasma proteomic risk markers for malignant ventricular arrhythmias and sudden cardiac death

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Background

Sudden Cardiac Death (SCD) was responsible for 70,000 deaths in the UK in 2010 and has been shown to have an incidence of 1 in 1,000 person-years. While preventative measures such as antiarrhythmic drugs and Implantable Cardioverter Defibrillators (ICDs) exist, current risk markers can miss those at risk or misattribute risk, leading to expensive, unnecessary surgery.
While plasma is a well-known source of biomarkers, the huge dynamic range of proteins means it is difficult to identify and quantify potential biomarkers and determine their association with disease. Although methods like immunodepletion can address this, they require significant sample material and expense.

Methods

Undepleted plasma samples (n=70) from healthy controls, patients with ICDs who suffered arrhythmic events and patients with ICDs who suffered no arrhythmic events were prepared using a novel two-pronged sample preparation method. Prepared samples were analysed with nano-LC-MS/MS using a Waters Synapt G2S instrument. Data analysis was carried out using ProteinLynx Global Serve 3.0 and Progenesis QI, along with a range of datamining software packages.

Results

In total over 4,800 proteins were identified, of which over 2,400 were quantifiable. Statistical and functional analyses of the protein and peptide data were carried out to generate a panel of risk marker candidates. Three predictive markers including cardiac cytoskeletal proteins and proteins previously associated with related cardiovascular diseases were ultimately selected. Individually, AUROCs of 0.11, 0.27 and 0.74 were observed for the proteins and following logistic regression a combined AUROC of 1.00 was observed. A targeted Selected Reaction Monitoring (SRM) workflow is in development to verify these findings in two verification patient cohorts.

Conclusions

These findings could prove invaluable to improve stratification of risk of SCD, leading to improved patient outcomes and reduction of the financial burden of unnecessary ICD implantation.

Keywords

Sudden Cardiac Death, SCD, Plasma, Proteomics

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Proteomic Assessment of HIV-Tat Treated Human Neurons: Towards a Molecular Model for HIV-associated Neurocognitive Disorders

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Background
A significant proportion of HIV-positive individuals are affected by the cognitive, motor and behavioral dysfunction that characterizes HIV-associated neurocognitive disorders (HAND). While the molecular aetiology of HAND remains largely uncharacterized, HIV transactivator of transcription (HIV-Tat) is thought to be an important aetiological cause.
Methods
We utilised shotgun mass spectrometry to quantify proteomic changes that occur when differentiated human neurons (derived from non-transformed human neuroepithelial stem cells) are treated in vitro with HIV-Tat. We analysed differentiated neurons by proteomic mass spectrometry at 6, 24, and 48 hours following treatment with recombinant HIV-Tat. Patch-clamp electrophysiology was also performed to functionally assess treated neurons.
Results
We identified over 4000 protein groups with 131, 118, and 45 protein groups differentially expressed at 6, 24, and 48 hours' post treatment, respectively ($p<0.05$). Alterations in the expression of proteins involved in gene expression and cytoskeletal maintenance were particularly evident. Proteins involved in cytoskeletal maintenance and transport (such as CTNA1, KLC2, and ARP10) exhibited significantly decreased expression over time. In tandem with proteomic evidence of cytoskeletal dysregulation; we observed statistically significant HIV-Tat induced functional alterations, including a reduction of neuronal intrinsic excitability as assessed by patch-clamp electrophysiology.
Conclusions
Our findings indicate that HIV-Tat may influence gene expression as well as structural elements of neurons. These effects may be involved in the aetiology of HIV neurodegeneration. Short term exposure to HIV-Tat also affects neurons on a functional level, as evidenced by reduction in neuronal excitability. This study identifies potential molecular mechanisms of HAND and forms the basis for further investigation of the proteins involved in aberrant gene expression and cytoskeletal maintenance, as well as long term assessment of HIV-Tat exposure (in progress).
Keywords
Neuronal cell culture, HIV-Tat, proteomic mass spectrometry, whole-cell patch clamp, HIV-associated neurocognitive disorders

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Possible causal link between insulin signaling deregulation and its etiology of aggressive prostate cancer

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The systems interrogation of human derived clinical specimens has now become requisite to any comprehensive biomarker discovery and its functional validation research program. It relies heavily on the analysis of high throughput functional genomic features at multiple levels of molecular biology events, namely gene expression at the transcript, protein and metabolite levels. Many of these biomolecular species are oftentimes manifested in exosomes and lipid microvesicles and exhibit both organotypic and organotropic properties. However, the clinical exploitation of such multi-omic observations, either in terms of gaining a mechanistic understanding of the molecules undergoing perturbations as they relate to specific disease processes, or how they effectively stratify disease down to the individual patient level (personalized medicine) regardless of their innate heterogeneity of presentation, has had limited success. To achieve this, we have developed a unique, high-precision LC-MS based, biomarker discovery and functional validation pipeline that can differentiate benign versus aggressive prostatic disease at the minimally invasive blood level. The functional validation of key differentiated protein was achieved with micro-RNA analysis against the same serum and tissue matched specimens. Multipsrametric non-linear regression modeling was used to derived the candidate biomarker panels. It was found that the progression to aggressive prostate cancer implicated the disruption of multiple biological pathways such as DNA damage response elements, autophagy, apoptosis, cell metabolism and their convergence to insulin signaling dysregulation. Hallmark proteins to these signatures included pyruvate kinase M1/M2 isoforms, p21, eEF isoforms 1-3, IGF BP isoforms 1-6, and MAP kinase isoforms 1 and 2 that could be used on a combinatorial basis for the early prediction of aggressive prostate cancer and its potential treatment.

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pTyr-phosphoproteomics of serial tumor biopsies from patients with advanced cancer treated with protein kinase inhibitors

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Background Mass spectrometry-based phosphoproteomics of tumor tissue lysates provides a potential personalized medicine approach based on its global information on aberrantly activated signaling pathways and potential drug targets. Here, we report results of a clinical trial (NCT01636908) to evaluate the effect of protein kinase inhibitor (PKI) treatment on tyrosine (pTyr) phosphoproteomic profiles of serial tumor needle biopsies in patients with advanced solid tumors.
Methods Tumor biopsies from 31 patients with advanced cancer were obtained before and after 2 weeks treatment with sorafenib (SOR), erlotinib (ERL), dasatinib (DAS), vemurafenib (VEM), sunitinib (SUN) or everolimus (EVE). Using matched protein input for paired biopsies per patient, down-scaled phosphopeptide immunoprecipitation was performed (P-Tyr-1000, PTMScan®) followed by LC-MS/MS (Q Exactive™) measurement. MaxQuant was used for phosphopeptide/site identification and quantification; fold-changes (Fc) were determined per biopsy pair. Tumor drug concentrations ([PKI]) were determined by LC-MS.
Results For pTyr-phosphoproteomics, biopsy pairs were profiled with median protein input of 2.0 mg per biopsy (range 0.8-2.9) for SOR/ERL/DAS cohorts and 1.1 mg (0.5-2.0) for VEM/SUN. Per biopsy, on average 440 ± 97 phoshopeptides were identified in SOR/ERL/DAS and 178 ± 64 in VEM/SUN, respectively. Unsupervised clustering based on phosphopeptide intensities showed clustering of biopsies from individual patients. Supervised clustering based on differential phosphopeptides (Fc > 5 in ≥ 3/5 patients per cohort) separated pre- and on-treatment groups. Differentially expressed phosphopeptides were drug-specific. Inhibition of (target) kinase activities related to clinical response was observed. Median [PKI] for SOR, ERL, DAS, SUN and EVE ranged between 2-10 µM, while median [VEM] was 1326 µM.
Conclusions In this study we were able to detect, for the first time, in tumors from patients treatment-induced specific changes in the phosphoproteome related to drug concentrations. This approach improves our understanding of individual tumor biology and will enable development of phosphoproteomics-based personalized medicine.

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Reprogramming of the acute translatome and total proteome underlies oncogene-induced stress adaptability

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Deciphering the key mechanisms that distinguish tumor cells from non-tumor cells is important for uncovering effective therapeutic targets. Previous studies have revealed that tumor cells have unique survival capacities in the GILA (growth in low attachment) condition, a stressful 3D culture condition in which non-tumor cells undergo apoptosis and gradually be eliminated. However, the underlying molecular mechanism remains elusive. Since acute changes in mRNA translation has been shown to represent a major component of stress adaptation, we hypothesized that tumor cells adapt to the GILA adversity via the acute synthesis of specific proteins compared with non-tumor cells. Using Click technology coupled with pulsed-SILAC and Mass Spectrometry, the global nascent protein translation profiles were compared between a non-transformed cell line and two oncogene-transformed cell lines under the GILA condition. Moreover, the impact of acute translatome rewiring on the global proteome was evaluated using multiplex Tandem Mass Tag labeling technology. These systematic analyses have uncovered three signatures of transformed cells under the GILA adversity, i.e. an Acute translatome signature, a Global proteomic signature, and an Adaptive proteomic remodeling signature. These signatures indicate systematic alterations of multiple biological processes in transformed cells under the GILA stress, including multiple key components of the membrane trafficking pathway, such as Rabgef1, Sec23b, Myo1b, and the Rab family proteins. In support of the role of this pathway in exosome biogenesis, transformed cells had increased exosome formation capacity compared with non-transformed cells. Importantly, both the conditioned media and the exosomes derived from transformed cells could confer non-transformed cells with adaptability in the GILA condition. Moreover, blocking Rab27b, one of the key regulators of exosome formation, diminished the stress adaptability and tumorigenicity of transformed cells. Collectively, our findings suggest that oncogenes systematically reprogram the membrane trafficking pathway to promote the unique stress adaptation of transformed cells and drive tumorigenicity.

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Characterization of site-specific N-glycoproteins in DBS sample by LC-MS/MS with minimal sample preparation

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Dried blood spot (DBS) samples have a number of advantages, especially with respect to ease of collection, transportation, and storage, and to reduce biohazard risk. N-glycosylation is a major post-translational modification of proteins in human blood that is related to a variety of biological functions, including metastasis, cell-cell interactions, inflammation, and immunization. Here, we directly analyzed tryptic N-glycopeptides from glycoproteins in DBS sample using liquid chromatography tandem mass spectrometry (LC-MS/MS) without centrifugation of blood sample, depletion of major proteins, desalting of tryptic peptides, and enrichment of N-glycopeptides. Using this simple method, we identified a total of 41 site-specific N-glycopeptides from 16 glycoproteins in the DBS sample, from immunoglobulin gamma 1 (IgG-1, 10 mg/mL) down to complement component C7 (50 μg/mL). Of these, 32 N-glycopeptides from 14 glycoproteins were consistently quantified over 180 days stored at room temperature. The major abundant glycoproteins in the DBS sample were IgG-1 and -2, which contain 9 asialo-fucosylated complex types of 16 different N-glycopeptide isoforms. Sialo-non-fucosylated complex types were primarily detected in the other glycoproteins such as alpha-1-acid glycoprotein 1, 2, alpha-1-antitypsin, alpha-2-macroglobulin, haptoglobin, hemopexin, Ig alpha 1, 2 chain C region, kininogen-1, prothrombin, and serotransferrin. We first report the characterization of site-specific N-glycoproteins in DBS sample by LC-MS/MS with minimal sample preparation.

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Contribution of combined limited proteolysis and LC-MS/MS to emerin self-assembly characterization

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Emerin is one of the inner membrane proteins anchored at the metazoan nuclear envelope. Its gene was discovered in 1994 because it is mutated in patients with X-linked Emery–Dreifuss muscular dystrophy (EDMD; Bione et al. 1994). Emerin was shown to interact with proteins playing a role in cytoplasmic and nuclear structure, as well as in chromatin organization. Emerin nucleoplasmic region, which is mostly intrinsically disordered, was revealed to self-assemble forming filaments in in vitro experiments. It was proposed that this property could regulate nucleoskeleton binding and that its deficiency could contribute to emerin loss of function (Herrada et al. 2015).
In order to increase the understanding of emerin functional regulation through self-assembly, the structural characterization of emerin oligomers was performed. Mass spectrometry was used to complement the Nuclear Magnetic Resonance and fluorescence spectroscopy analyses (Samson et al. 2017). We here present the approach combining digestion by proteases and liquid chromatography mass spectrometry to analyze different emerin oligomers.

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High Throughput sensitive microLC-MS/MS for Peptide Quantitation in highly targeted assays

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Background

For Research Use Only. Not for Diagnostic Procedures

Analysis of therapeutic proteins and protein biomarkers is increasingly performed by LC-MS/MS, using signature tryptic peptides for quantitation. Sample preparation can consist of immunocapture at the protein level, or enrichment at the peptide level. To further increase sensitivity for low abundant proteins, microflow LC-MS can be used instead of traditional LC-MS to achieve sensitivity improvements previously demonstrated of up to 5x. In this paper we describe a robust trap-and elute microLC method with a total cycle time of 3 minutes, that can be used for samples containing up to 5 signature peptides and their isotopically labeled standards.

Methods

The system used consisted of a NanoLC™ 425 dual gradient system, coupled to a QTRAP® 6500 system (SCIEX) in MRM mode. Using a gradient loading pump, sample is loaded on a 0.5 x 10 mm ChromXP C18 cartridge column (SCIEX) at 80 µL/min. Any non-binding matrix components are washed to waste. The trap column is then switched on-line with the MS, and the peptides are eluted isocratically in 15 seconds for quantitation. The trap is then switched back again, and washed to remove any remaining bound matrix components and detergent added during sample preparation.

Results

Using the above described 3 minute method, precision at concentrations of 1 fmol/µL or higher was 3-6% CV, and accuracy was within 15%. Carryover was <0.5%. The method will be used for the quantitation of proteins in plasma samples, processed using the SISCAPA sample preparation method.

Conclusions

A robust, very high throughput microLC method for sensitive quantitation of limited numbers of peptides in immunoaffinity enriched samples has been developed. This method can be particularly useful for the quantitation of biologics and peptide or protein biomarkers.

Keywords

Biologics, Biomarkers, Peptide Quantitation, Protein Quantitation, Microflow LC/MS

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Brain metastatic cancer cells mimicking neurons: a proteotranscriptomic analysis.

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Brain metastases are a feared outcome of cancer. At this stage, there is no way to predict which tumors will metastasize to the brain and no specific treatment for brain metastasis. However, it has been described that some neuronal biomarkers (such as GABA receptors) may be increased in cancer cells that metastasize to the brain. In the present study, the expression of neuronal proteins and mRNA was analysed in the MDA-MB-231 vs 231-BR human breast cancer model, where 231-BR cells are derived from MDA-MB-231 and metastasize specifically to the brain. SILAC based quantitative proteomics and microarray transcriptomics were previously performed by our group (1) and here we have specifically extracted mRNA and proteomic data on membrane and secreted neuronal proteins. Interestingly, a significant proportion of neuronal proteins, including synaptic vesicle membrane proteins, neuronal calcium sensors or neuropilin receptors, were found in breast cancer cells with some significantly increased in the brain metastatic 231-BR cells. Ingenuity Pathway Analysis revealed modifications in signaling networks related to neural plasticity and metabolism. Protein changes were not always matched with changes in corresponding mRNA, suggesting significant posttranscriptional regulations. In addition, we have detected some of these neuronal proteins by immunohistochemistry in primary breast tumors as well as in other human malignancies; they may constitute novel biomarkers for prognostication of brain metastasis. Together, this exploration of the neuronal proteome of breast cancer cells shows that refining omics data sets with an hypothesis driven approach is a productive strategy, and that brain metastatic cancer cells exhibit a neuronal phenotype.

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Ultra-deep palmitoylomics enabled by a dithiodipyridine functionalized magnetic nanoparticles

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Palmitoylation, a type of fatty acylation, has vital roles in a wide range of biological processes by regulating proteins' membrane tethering. Here we presented a strategy for systematic identification of protein palmitoylation using a novel dithiodipyridine functionalized magnetic nanoparticles-based enrichment approach. We identified 1309 putative palmitoylated proteins from mouse brain tissues, among which 883 proteins have not been previously suggested to be palmitoylated in previous proteomic studies. Especially, we validated the palmitoylation of GSK-3β and found that the cellular localization and biological functions of GSK-3β was regulated by palmitoylation. Therefore, our method based on Fe_3O_4/SiO_2-SSPy microspheres allows for markedly deeper coverage of palmitoylome due to their high surface-to-volume ratio with abundant reactive functional groups and low non-specific adsorption.

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Quantitative Proteomics Analysis of a Primary Bladder Cell line treated with Ketamine

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Ketamine abuse has increased rapidly over the past years and is one of the most abused psychotropic substances in many parts of the world. A syndrome of cystitis and contracted bladder can be associated with ketamine abuse. Secondary renal damage can occur in severe cases which might be irreversible, rendering patient's dependent on dialysis. There is an urgent need to study the mechanisms of ketamine-induced ulcerative cystitis and psychological dependence to promote effective treatments for these ketamine-related disorders. Ketamine affects diverse cellular pathways, however, the ketamine -induced alterations on the global proteome across human cell lines have not been fully elucidated. To explore the molecular mechanism underlying ketamine cystitis, we used the iTRAQ-based LC-MS/MS technology to systemically quantify the levels of proteins in a primary bladder cell line treated with ketamine at different time intervals. More than 3500 proteins were quantified from the total cellular proteome; among them, >120 proteins were found to be significantly up- or down-regulated after ketamine treatment. Network analysis revealed the involvement of these proteins in biological processes related to immune response, cytoskeleton remodeling, cell adhesion and blood-coagulation pathways. Our study provides valuable information regarding the global effect of ketamine on protein expression of human bladder cells, which can be very helpful for understanding the molecular mechanism of ketamine-induced cystitis.

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Lung cancer biomarker discovery using integrated transcriptomics and proteomics approach

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Background
Lung cancer is the most common cause of cancer related death worldwide. The 5-year survival rate is 17.5 % according to figures from Korean National Statistical Office. The prognosis of lung cancer patients is very dependent on how advanced their disease is. In stage I, 5-year-survival rates are about 70%, whereas survival rate is about 1% in stage IV. Therefore, early detection of lung cancer would increase the survival rate. We performed a transcriptome-proteome integrated assay to discover novel biomarker for lung cancer diagnosis.

Methods
We performed RNA sequencing (RNA-seq) in 5 lung cancer and adjacent normal tissue pairs to identify differentially expressed genes (DEGs). Based on acquired transcriptomics data, 757 up-regulated genes / 688 down-regulated genes were selected. Then, we obtained biomarker candidates shown proteomic expression evidence using Mass Spectrometry spectral library and plasma proteome database (PPD). A total of 64 proteins were monitored through the skyline spectral library to establish MRM methods and finally 40 proteins were alive for the next MRM approach. MRM mode was achieved on ten pooled plasma and tissues acquired from patients with/without lung cancer for target screening using Q-TRAP 5500.

Results
A total of 99 DEGs were selected (Fold change> 2, $p<0.05$, FPKM> 5) from transcrptomics data of lung cancer tissue and adjacent normal tissue pair. Among them, 64 proteins expected to be present in plasma were selected using PPD. Among them, MRM methods of 40 genes were predicted using Skyline v3.6 and then performed MRM-MS on plasma/tissue samples. A total of 22 proteins altered in lung cancer tissue were finally selected for the further clinical validation with MRM-MS.

Conclusions
We obtained 22 biomarker candidates for lung cancer using integrated analysis of transcriptomics and proteomics and determined the protein levels of these candidates in clinical samples.

Keywords
Transcriptomics, Proteomics, Lung cancer, Biomarker

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Therapeutic target discovery by cancer proteomics
in the era of large-scale genome-resequencing

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Recent large-scale sequencing efforts by the TCGA and others have identified a high frequency of mutation (>90%) of genes in the Wnt signaling pathway in colorectal cancers. However, only a few technically "druggable" molecules (secreted proteins, cell surface receptor proteins, and kinases/enzymes) have been found in the pathway.

We adopted a comprehensive proteomic approach to identify a new druggable target in the Wnt signaling pathway. Nuclear proteins of two colorectal cancer cell lines, HCT-116 and DLD1, immunoprecipitated with anti-TCF-4 antibody were analyzed directly by nano-flow liquid chromatography (LC) and tandem mass spectrometry (MS/MS).

We identified TRAF2 and NCK-interacting protein kinase (TNIK) as one of 70 components of the TCF-4/β-catenin transcriptional complex, the most downstream effector of Wnt signaling [1]. TNIK was essential for the transactivation of Wnt target genes and the proliferation of colorectal cancer cells. Knockdown of TNIK inhibited the growth of tumors produced by subcutaneous injection of colorectal cancer cells into immunodeficient mice [2]. TNIK-deficient mice were resistant to azoxymethane-induced colorectal tumorigenesis and developed significantly fewer intestinal tumors.

Through a screen of a kinase-focused compound library, we have recently identified a novel small-molecule compound that inhibits the kinase activity of TNIK with an IC50 value of 21 nM. The compound inhibited Wnt signaling and the sphere- and tumor-forming activities of colorectal cancer cells. The compound was orally administrable and significantly suppressed the growth of patient-derived colorectal cancer xenografts [3]. Based on these findings we consider the compound to be a potential candidate for clinical trials [4].

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A comprehensive analysis of glycome profiles on formalin-fixed paraffin-embedded mouse tissue sections

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Background: In our previous work, we have established a simple and reproducible method for high-throughput and in-depth glycomic profiling of formalin-fixed paraffin-embedded (FFPE) tissue sections using laser microdissection (LMD) and lectin microarray (LMA) (Zou et al. Sci. Rep. 2016). Using this approach, we have successfully revealed characteristics in glycomic profiles of morphologically different parts in five mouse organs including brain, liver, kidney, spleen, and testis. Here, we evaluated whether this standardized method could apply to other mouse tissues.

Methods: FFPE sections of nine tissues including thymus, lung, heart, pancreas, gall bladder, stomach, small intestine, colon, and skin from two 8-week-old male C57BL/6J mice were additionally adopted in this study. Tissue fragments with different morphology were collected from hematoxylin-stained sections by LMD. The proteins extracted from these fragments were Cy3-labeled and subjected to LMA with 45 lectins.

Results: A pilot study confirmed that tissue fragments of as small as 5 µm × 0.9 mm^2 were sufficient for LMA analysis of the nine tissue sections. In total, LMA data of 234 size-adjusted tissue fragments were additionally collected from the nine tissue sections. Multivariate analysis on glycome data obtained from a total of 14 tissues (previously analyzed 5 tissues and currently analyzed 9 tissues) clearly revealed variations of glycome profiles in different tissues and in different parts of one tissue, which was partly confirmed by lectin histochemistry.

Conclusions: Our standardized method with combined use of LMD and LMA can apply to FFPE tissue sections of the whole body, providing comprehensive tissue glycome mapping data to overlook cell-, site-, and tissue-specific glycosylations. Since aberrant glycosylation under pathophysiological states has been observed in many diseases including cancer, the present differential glycomic profiling strategy will also serve as a triggering item for discovery of disease-related glyco-biomarkers.

Keywords: lectin microarray, laser microdissection, mouse tissue, glycome mapping

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A pathogenic Escherichia coli virulence factor manipulates the host ubiquitin-modified proteome during infection

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Enteropathogenic Escherichia coli (EPEC) causes severe diarrheal illness worldwide. To cause disease, EPEC utilizes a type III secretion system (T3SS) to inject toxic effector proteins into human intestinal epithelial cells. Effectors typically possess enzymatic activity, enabling EPEC to directly post-translationally modify host proteins and subvert cellular processes, thereby causing disease. However, EPEC possesses dozens of effectors, each with unique roles, making it challenging to identify mechanistic events that contribute to disease. Previously, co-immunoprecipitation and yeast two-hybrid techniques were used to identify effector targets; however, these methods fail to capture global consequences of effector activities. Understanding the global consequences of infection on the human proteome and post-translational modifications would provide a greater understanding of how T3SS effectors contribute to disease. Furthermore, unbiased approaches can simultaneously identify direct substrates of enzymatically active effectors.

This study aims to identify substrates of the EPEC ubiquitin ligase NleG and simultaneously characterize the global impact of NleG and EPEC infection on the host ubiquitin-modified proteome by enriching for the ubiquitin-modified proteome. Cultured human epithelial cells were differentially labelled using stable isotope labeling by amino acids in cell culture and infected with wild-type EPEC or a strain lacking nleG. The ubiquitin-modified proteomes of infected cells were compared using tandem mass spectrometry and analyzed by Mascot and MaxQuant. We identified 20 proteins differentially ubiquitinated in the presence of NleG and confirmed a novel host substrate implicated in the regulation of innate immune signaling and cell death, which may provide a mechanism by which NleG contributes to disease. Preliminary analyses also identified pathways affected by NleG that are known to be targeted by EPEC, including innate immunity and cytoskeletal regulation. These pathways implicate NleG in several infection-mediated impacts on disease-related pathways and demonstrate the value to applying ‘omics technologies to study infection.

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Proteomics analysis of historical collagen glue retrieved from a Stradivari violin

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Background: Protein-based glues were the most common adhesives used by European craftsmen before the industrial revolution. While studying the wood properties of a Stradivari violin, we encountered an unknown glue that was applied for its neck modification carried out around 1800.

Methods: We applied a simple procedure of direct-contact trypsinization for solid glue and ZipTip cleanup, and analyzed the digested peptides by UPLC-ESI-QTOF-MS/MS. Peptide identification was carried out by MASCOT search against many different animal species in NCBI databases.

Results: The profile of identified peptides showed a high abundance of type I collagen. One of the chief concerns about this analysis was that chemical damage over 200 years may render the protein unidentifiable. However, our results showed the contrary, as the historical glue yielded more identifiable peptides than modern glues digested similarly. Collagens form triple helices which are then covalently crosslinked by enzymatic actions, which may interfere with trypsin digestion. The chemical damage suffered by the historical glue may have resulted in backbone cleavage and therefore de-crosslinking, facilitating trypsin digestion. Searching against NCBI databases, the collagen sequence of the historical glue matched the Bovidae family, but further differentiation between cow, sheep, and goat collagens was challenging due to high sequence homology. Analysis of modern glues used by violin makers also revealed that some cow glues are mislabeled as rabbit glues on the market.

Conclusion: Our results showed that UPLC-ESI-QTOF-MS/MS and MASCOT search could be easily applied to the analysis of historical glues, with even better results than modern glues.

Keywords: collagen, glue, Stradivari violin, trypsin digestion

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Identification of biomarkers for Proliferative Diabetic Retinopathy (PDR) in the human vitreous humor

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Background: Diabetic retinopathy (DR) is the damage caused to the retina due to diabetes. DR can be either non-proliferative (NPDR) or proliferative (PDR). The pathology of PDR includes capillary occlusion, neovascularisation and breakdown of the blood-retinal barrier, which will eventually result in the loss of vision. Although the prevalence of diabetic retinopathy among self-reported diabetics is 26.8%, in most cases diagnosis is achieved only at an advanced stage. Biomarkers for early detection of PDR will tremendously help in the management of this disease. As the pathological conditions of the retina in PDR will affect the protein components of the vitreous, we performed a quantitative proteomic analysis of vitreous to identify differentially regulated proteins in PDR vitreous.

Methodology: Vitreous samples of PDR patients and non-diabetic subjects were used. The abundant proteins in the samples were depleted using a Human 14 Multiple Affinity Removal System. The iTRAQ labelled samples were fractionated using SCX chromatography and analysed on a LTQ-Orbitrap Velos mass spectrometer. The mass spectrometry data was searched using SEQUEST through Proteome Discoverer software suite. Validation studies were carried out using Multiple Reaction Monitoring (MRM) and ELISA.

Results: We identified a total of 677 proteins, out of which 63 were found to be more abundant while 76 were found to be less abundant in PDR vitreous when compared to normal vitreous. Several proteins that play a role in angiogenesis and vascular permeability were identified. Overabundance of A2M, ABCB9, AMBP, COL1A1, and FGG were validated in twelve PDR vitreous samples using LC-MRM and ELISA.

Conclusions: This study provides a number of proteins which are found to be overabundant in the vitreous of PDR patients. Further, validation of these differentially regulated molecules in an easily accessible fluid such as tear will lead to the development of biomarkers for early detection of diabetic retinopathy.

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Proteomic study on advanced glycation end-products treatment in kidney of mice

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Diabetic nephropathy is the most common cause of end-stage renal disease in the world. Advanced glycation end products (AGEs) are heterogeneous cross-linked sugar-derived proteins which could accumulate in glomerular basement membrane, mesangial cells, endothelial cells, and podocytes in patients with diabetes and/or end-stage renal failure. AGEs are thought to be involved in the pathogenesis of diabetic nephropathy via multifactorial mechanisms such as oxidative stress generation and overproduction of various growth factors and cytokines. This study aims to analyze advanced glycation end-products (AGEs)-mediated protein network in mice kidneys. We used mass spectrometry to detect proteome in kidney from streptozotocin (STZ)-induced diabetic mice kidneys. Many of proteins are functionally associated with kidney toxicity and specific mitochondrial dysfunction related proteins were identified in AGEs-treated mice kidney. Moreover, we found grade 1 metastatic necrosis of renal tubules without inflammation in AGEs-treated mice kidney. AGEs are likely to induce diabetic nephropathy by inducing chronic mitochondrial dysfunction in the mice kidney tubules.

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Development of Online 2D-NCFC-RP/RPLC System for Extensive and Efficient Proteomic Analyses.

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Background

Proteomics aims to complete profiling of the protein content and their modifications in cells, tissues, and biofluids and to quantitatively determine changes in their abundances. This information reveals cellular processes and signaling pathways and serves to identify candidate protein biomarkers and/or therapeutic targets. Therefore, analysis should be comprehensive and efficient.

Methods

Here, we present a novel online two-dimensional reverse-phase/reverse-phase liquid chromatography separation platform, based on a newly developed online non-contiguous fractionating and concatenating device (NCFC fractionator).

Results

In bottom-up proteomics analyses of a complex proteome, this system provided significantly improved exploitation of the separation space of the two RPs, resulting in a considerable increase in the numbers of peptides identified compared to a conventional contiguous 2D-RP/RPLC method.

Conclusions

The fully automated online 2D-NCFC-RP/RPLC system bypassed a number of labor-intensive manual processes (offline fractionations, poling, clean-up, drying/reconstitution, and autosampler fraction injection) required with previously described offline 2D-NCFC-RP/RPLC method, offering minimal sample loss and highly reproducible 2D-RP/RPLC experiments.

Kewords

Proateomics, online 2DLC, NCFC

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Detection of Plasma Colorectal Cancer Prognostic Biomarkers

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Background: Human plasma arguably contains one of the most complex human proteomes and possesses the ability to reflect progress of human diseases, potentially including early detection of cancers. Colorectal cancer (CRC) is a particularly devastating condition, claiming ~700,000 lives/year. Although early detection significantly increases survival, it is really detected at early stage. This study aims to build technologies (SWATH-MS) that are potentially capable of identifying plasma prognostic biomarkers for early CRC detection.

Methods: To maximise CRC plasma protein identification from SWATH plasma library, 14 highest abundant proteins (HAPs) were depleted from pooled staged CRC plasmas. Depleted plasmas were tryptically digested and fractioned using 4 different independent methods (high pH reversed-phase, strong anion exchange, strong cation exchange and size exclusion chromatography). Quantitative SWATH-MS analyses were performed on 100 CRC patient plasma pools (n=20 patients in each of CRC AJCC stages I-IV and age- and sex-matched healthy controls). SWATH-MS was performed on both depleted (14 HAP) and non-depleted plasmas.

Results: A total of 529 unique, high-quality, plasma proteins were identified from 4 different peptide fractionation methods with 1% FDR level. Out of these, 363 proteins were quantified from depleted CRC plasma and 315 from non-depleted plasma. Statistical analysis (BH-adjusted p-value <0.05 and fold change >1.5) showed 4 protein candidates exhibited differential expression across all CRC stages compared to healthy controls from both depleted and non-depleted samples. Two candidates (S10A8 and SAA2) have already been proposed as plasma biomarkers for CRC. We are currently validating these and other 2 candidates.

Conclusions: Our analysis revealed 2 known prognostic candidates effectively validating the methodology employed. We also uncovered 2 novel candidates. We are extending SWATH-MS analysis to the 100 individual CRC and control plasmas to identify biomarkers that associate with CRC patient early stage disease and with clinical outcomes (e.g., survival, recurrence and chemotherapy effectiveness).

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Phosphoproteomics study reveals the regulation of vasodilator-stimulated phosphoprotein upon stathmin-1 silencing in colorectal cancer metastasis

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Background
Stathmin-1 plays a crucial role in cancer metastasis, and is a potential target in anti-metastatic therapy for colorectal cancer patients. In this study, we present the results in the changes in the phosphoproteome in an isogenic pair of colorectal cancer cell lines, the poorly metastatic HCT-116 and the highly metastatic derivative E1 upon stathmin-1 knockdown. The main aim is to better understand how alterations in phosphoproteins are involved in colorectal cancer metastasis.

Methods
The phospho-peptides of stathmin-1 knockdown E1 and HCT-116, and their control cells were enriched via the TiO2 HAMMOC approach. Comparative quantitative proteomics analysis of the phospho-peptides was carried out using the label-free SWATH-MS technology.

Results
Bioinformatics analysis identified a list of phospho-proteins that are regulated in response to stathmin-1 silencing, and many of these are involved in signaling, cytoskeletal structure, and focal adhesion. Among these phospho-proteins, alterations in phosphorylation status of the actin cytoskeleton regulator, vasodilator-stimulated phosphoprotein (VASP) was found to be concomitant with stathmin-1 associated metastatic potential of the cancer cell lines. We thus showed that silencing of stathmin-1 alters the expression, subcellular localization and phosphorylation status of VASP, which might be associated with remodeling of cytoskeleton in cancer metastasis.

Conclusions
This phosphoproteomic study showed that the downstream effects of stathmin-1 knockdown involved the cytoskeletal, focal adhesion and signal transduction proteins. These targeted effects of stathmin-1 silencing supports the role of stathmin-1 as a target for cancer metastasis. We further proposed that stathmin-1 knockdown regulates VASP, which could enhance the inhibition of colorectal cancer metastasis.

Keywords
Colorectal cancer metastasis; Phosphoproteomics; Stathmin-1; Vasodilator-stimulated phosphoprotein; SWATH-MS

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Quantitative proteome analysis of VEGF-induced vascular hyperpermeability in a mouse model by LC-MS/MS

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Background
Vascular endothelial growth factor (VEGF) is known as vascular permeability factor which cause dysregulation of junction integrity between endothelial cells and resultant vascular leakage. Also, retinal vascular hyperpermeability cause macular edema which leads to visual deterioration and various retinal disease such as diabetic retinopathy. Accordingly, anti-VEGF agents have been used to treat retinal vascular hyperpermeability. However, VEGF plays an important role in the survival of normal endothelial and neuronal cells in the retina and because anti-VEGF has potential toxicity, the goal of this study is to identify novel therapeutic targets for retinal vascular hyperpermeability.

Methods
We performed quantitative proteome profiling analysis on mice of three conditions (Control, VEGF, VEGF plus Anti-VEGF group). We used the iTRAQ (Isobaric tag for relative and absolute quantitation) for labeling on the peptide after modified FASP (Filter Aided Sample Preparation) digestion. The iTRAQ labeled peptide samples were divided into 24 fractions by using a mid pH reverse phase liquid chromatography and each fraction was individually analyzed by LC-MS/MS experiments.

Results
We identified a total 205,730 non redundant peptides, 8,685 proteins with 479 DEPs. Among them, 416 DEPs (283 up-regulated and 133 down-regulated) in VEGF-treated samples, compared with the control samples (VEGF versus control); and 314 DEPs (86 up-regulated and 228 down-regulated) in the VEGF plus anti-VEGF-treated samples, compared with VEGF-treated samples (anti-VEGF versus VEGF).

Conclusions
β2 integrin was up-regulated by VEGF treatment, but the alteration was inhibited by cotreatment of VEGF and anti-VEGF antibody which also confirmed by western blotting analysis. Finally, we experimentally demonstrated that β2 integrin can serve as an effective therapeutic target for retinal vascular hyperpermeability.

Keywords
Retinal tissue, β2 integrin, VEGF, iTRAQ, LC-MS/MS

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Mass spectrometric analysis of complete cellular proteome via direct digestion of cells

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Background
Mass spectrometry-based proteomics is widely employed in the field of biological research. However, the conventional sample preparation workflow is tedious and time-cost, which make it difficult for researchers without professional training of proteomics related techniques. Therefore, it is of great importance to develop simplified protocols for proteomics analysis.
Methods
Inspired from the use of trypsin in cell lysis. We built up a novel method, named the cell-absorb method, for mass spectrometric analysis of complete cellular proteome. In brief, living cells were absorb into the vacuum dried gel pieces and then digested into peptides by trypsin directly.
Results
Benefited for the simplification of the workflow, the additional time for cell lysis and protein extraction was saved by the novel cell-absorb method. Compared with two tradition mehtods (the SDS-PAGE based method and the pro-absorb method), the cell-absorb method exhibite superority in terms of the number of proteins and peptides identified. Further analysis shew that the new method was advantageous in identifying large proteins.
Conclusions
Collectively, we developed a new method, termed the cell-absorb method, which was the first method that was capable for analyzing complete cellular proteome via direct digestion of cells in gel pieces.
Keywords
Mass spectrometry, protein, proteomics, cell, in-gel digestion

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OST3 proteins link glycosylation and magnesium transport

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Background;

N-glycosylation is an essential co- and post-translational protein modification that affects protein folding, stability and function. N-glycosylation is catalysed by an enzyme complex called oligosaccharyltransferase (OTase). Ost3p and Ost6p are homologous subunits of OTase and the orthologues in human are MagT1 and TUSC3. Both human proteins have been reported to be involved with N-glycosylation, while MagT1 has also been reported to be required for magnesium transport. We used a yeast model system to test this dual role of Ost3p and Ost6p in N-glycosylation and magnesium transport.

Methods;

We used a Saccharomyces cerevisiae Tetracycline-repressible system, where knockdown of gene expression is induced by the addition of doxycycline. This allowed us to simultaneously deplete both Ost3p and Ost6p in our yeast model. We grew yeast with or without doxycycline, and with and without magnesium supplementation. We analysed whole cell proteomes and cell wall glycoproteomes using SWATH workflows with a TripleTOF 5600 mass spectrometer.

Results;

Knockdown of Ost3p and Ost6p expression resulted in a substantial growth defect compared to wild type yeast, which was partially rescued by supplementation of magnesium to the growth medium. Cell wall glycoproteomic analyses showed that cells deficient in Ost3p/Ost6p had substantial defects in protein N-glycosylation at diverse sites. Although magnesium supplementation rescued growth, it did not rescue this glycosylation defect. Whole cell SWATH proteomics of Ost3p/Ost6p deficient cells in comparison to wild-type yeast showed down-regulation of proteins related to protein synthesis, and magnesium supplementation rescued the abundance of a subset of these proteins. To validate these findings, we cloned and over-expressed selected proteins identified by proteomics. This showed that over-expression of key Ost3p/Ost6p-magnesium transport dependent proteins rescued growth of Ost3p/Ost6p deficient yeast independently of N-glycosylation.

Conclusions;
Yeast Ost3p and Ost6p play dual roles as regulatory subunits of OTase, and in magnesium transport.

Keywords;
Yeast Proteomics, OTase, N-glycosylation

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Development of serum protein biomarkers for the recurrence in gastric cancers using multiple reaction monitoring

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Background
Despite improvements in clinical therapies of Gastric cancer (GC), the recurrence rate of GC patients remains high (~55%) with advanced stage of the disease. Therefore, it is essential to understand of GC recurrent mechanisms that would help effective clinical application for GC diagnosis and prognosis. Here, we aimed to identify potential serum biomarkers for monitoring the recurrence in gastric cancers with an established quantitative multiple reaction monitoring (MRM) approach using GC patient serum samples.

Methods
For global proteomic analysis, immunodepleted serum samples of various disease states were digested with trypsin and then analyzed by a Q-Exactive MS. The multiplexing MRM assay was performed on an Agilent 6490 QQQ MS with 105 target proteins of interest. MSstats analysis was carried out for translating chromatographic peak areas into log2 values and data normalization.

Results
To identify potential serum GC biomarkers for MRM analysis, we first performed an integrative biomarker candidate selection strategy using three independent approaches. By employing preliminary MRM results using GC patient serum samples, we established the quantitative MRM analysis for 105 proteins as serum biomarker candidates for monitoring the recurrence in gastric cancers. We further verified with independent patient cohorts encompassing 248 individual serum samples and identified that 12 potential biomarker proteins, IGFBP3, PROZ, F2, TF, PRDX1, TIMP1, TTR, HPX, NCAM1, ACTB, HBA1, and CR2, showing significant changes in abundance (p-value ≤0.005) in GC recurrent patient samples.

Conclusions
In this study, we have established a quantitative MRM assay for the identification of potential biomarkers monitoring the recurrence in gastric cancers. Although further studies are required to validate the panel of 12 potential serum biomarkers with a large cohort of patient samples, we propose that these proteins are considered as potential protein biomarkers to predict the recurrence in GC.

Keywords
Gastric cancer, Potential serum biomarker, Multiple reaction monitoring

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Using cross-linking mass spectrometry and integrated modeling to study architecture and dynamics of signaling complexes

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Introduction
The integration of hybrid methods to interpret complex structural assemblies has been a longstanding goal in molecular biology. Emerging methods in structural mass spectrometry (MS) can pose an exciting alternative to classical methods in structural biology, due to their low sample requirements and comparatively high measuring speed. We use such an MS based approach to study the architecture and dynamics of protein complexes involved in signaling.

Methods
We recently developed a generic hybrid approach for the modeling of large proteasomal assemblies that combines cross-linking MS with integrated modeling. Our approach employs a Bayesian scoring function and uses MS based information on spectral quality and frequency of detection as well as standard excluded volume criteria and sequence connectivity restraints as an input to evaluate and rank models. The best-scoring candidate models that most satisfied our cross-linking data are then grouped using the RMSD as a structural similarity criterion. The cluster of models with the higher population and lower average score can be chosen as the final solution set and represented by individual localization densities.

Results
This integrated platform is now used to study the architecture and structural dynamics of cyclic AMP-dependent protein kinase (PKA) complexes. This approach takes advantage of the fact that many proteasomal assemblies, in particular highly heterogeneous, very large or dynamic systems, remain refractory to structure determination by a single more conventional approach, but are in principle addressable by such a hybrid approach. We show for example that using quantitative crosslinking MS reveals the position of the dimerization and docking domain within a prototypical anchored PKA complex.

Conclusions & Novel Aspects
Structural MS and integrated modeling allow for the dynamical characterization of important signaling complexes

Keywords
Structural mass spectrometry, Cross-Linking, Integrated Modeling, Protein Complex

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AUTOANTIBODY PROFILING IN SCHIZOPHRENIA AFFECTED BRAIN TISSUES

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Background:
Schizophrenia affects approximately 1% of the word population and is considered as a major chronical mental illness. In the recent years studies have shown a correlation between higher levels of autoantibodies and the frequency of autoimmune disease in patients with schizophrenia compared to healthy individuals. In this study we used a targeted affinity proteomics approach to validate the autoantibody repertoire of brain tissue obtained from patients diagnosed with schizophrenia and compared these with healthy brain tissue.

Methods:
This study aims to identify a potential autoimmune reaction in the tissue of schizophrenia patients. In total we analysed the tissue from 125 individuals, including 73 schizophrenia patients and 52 controls. Based on previous in-house and external published studies of autoantibodies in psychosis, we selected 220 protein fragments from the Human Protein Atlas with a length of roughly 80 amino acids. Autoantibody profiling was performed using suspension bead array technology and IgG reactivity was measured in the tissue lysates from patients and controls.

Results:
Our findings indicate altered immune response in patients with schizophrenia compared to healthy controls. In our study we identified potential predictive autoimmune signatures. Among these we found autoantibodies targeting a protein subunit known to play a role in the context of schizophrenia, presented with higher IgG reactivity in patients compared to healthy control tissue."

Conclusions:
With our approach we were able to profile brain tissue of schizophrenia patients for possible autoantibody reactivity's. By further validating these putative autoimmunity targets, we could gain insights into the autoantigens associated to chronical mental illnesses.

Keywords:
Affinity Proteomics, Psychiatry, Microarray, Autoimmunity

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Dissecting the subcellular secretory glycoproteome with SWATH

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Background
N-glycosylation is a critical post-translational modification that influences the folding and function of half of the cellular proteome. The biosynthesis of N-glycoproteins begins in the endoplasmic reticulum (ER), where an oligosaccharide is transferred to selected asparagine residues in nascent polypeptides by the enzyme oligosaccharyltransferase. The presence of N-glycans at specific sites is critical for efficient productive protein folding in the ER, and defects in this process perturb glycoprotein folding, secretion, and function at a systems level. We have developed integrated subcellular fractionation and SWATH glycoproteomic workflows to understand the causes and consequences of changes in the N-glycosylation biosynthetic pathway.

Methods
We combined biochemical subcellular fractionation methods with quantitative SWATH-MS glycoproteomic and proteomic workflows to measure the response to a range of genetic and chemical perturbations to N-glycoprotein biosynthesis.

Results
We optimized biochemical fractionation methods in yeast to enable precise analysis of the subcellular proteome and glycoproteome. This enabled quantitative measurement of subcellular proteomes and site-specific and global profiling of glycan occupancy and structure. We tested these methods in yeast with defined defects in N-glycosylation, and then expanded our analysis to profile the quantitative effects of combined defects in glycoprotein biosynthesis and protein quality control on glycoprotein maturation.

Conclusions
Our results give key insights into the effect of site-specific glycosylation on glycoprotein quality control processes, and our methods will be useful in diverse applications in industrial and medical glycobiotechnology.

Keywords
N-glycosylation, SWATH, subcellular fractionation, glycoproteomics

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Towards a fit-for-purpose selection of sample preparation strategies for mass spectrometry-based proteomics

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Background: The selected workflow for a proteomics experiment is a key determinant for information that will be obtained, yet the corresponding selection is not always based on objective criteria and/or an educated evaluation. Here, we report a comparison of commonly-used proteomics sample preparation strategies which may provide guidance for a more rational selection of sample preparation strategies.

Methods: In-gel (IGD), in-solution (ISD), on-filter (OFD), and on-pellet digestion (OPD) workflows were compared on the basis of targeted (QconCAT-SRM method for mitochondrial proteins) and shotgun proteomics (DDA) analyses. In this study, three different otorhinolaryngological tissue samples (nasal polyps, parotid gland & palatine tonsils) were studied upon cryogrinding, and protein extraction in RapiGest (ISD) or SDS/urea lysis buffer (IGD, OFD & OPD) using 20 µg of protein as starting material and five replicates per condition.

Results: SRM and DDA data reveal comparable protein/peptide recoveries for all methods except for IGD (approximately 50-70% lower recovery), elevated imprecision for OPD in SRM experiments, and comparable precision for all methods following label-free quantitation (upon median normalization). Notable differences regarding the discovery potential of these workflows include: higher identification rates for ISD (and lowest for IGD), relatively more missed cleavages for ISD, more Met-oxidated & Asn-/Gln-deamidated peptides for IGD, and around 40% less Cys-containing peptides identified when using OFD. Furthermore, comparison of physicochemical properties (e.g. GRAVY, MW, pI) of identified proteins reveals some minor differences, yet the distributions of these properties as observed for the different approaches are in general rather comparable.

Conclusions: As the most suitable sample preparation approach may be different for different types of (clinical) samples or proteomics experiments, a fit-for-purpose workflow selection may be beneficial to achieve a higher potential of your proteomics studies.

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Identification of differential expression proteins in renal cell carcinoma by label-free quantitative proteomics analysis

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Background
Renal cell carcinoma is currently the second incidence of urinary tract cancer. In order to find the specific biomarker of renal cell carcinoma, we used proteomics techniques to study the differential expression proteins between renal tumor tissues and adjacent normal tissues.
Methods
Six Pairs of renal cell carcinoma and adjacent normal renal tissue were obtained from surgical resection and analyzed with QExactive Plus coupled with nanoLC system with a label-free quantitative approach. The differentially expressed proteins, such as PYGL、 ANXA4、 PSMB8、 CORONIN 1A , were validated by RT-PCR, Western blotting. The results were further validated in another cohort including 80 patients with Quantitative Dot Blot, which was recently developed for high throughput immunoblot validation. For in vivo validation, PSMB8, ANXA4 and PYGL were further knock-down in renal cell carcinoma cells （786-O）with shRNA. The cell proliferation and migration status of knock-down cells were investigated with xCELLigence Real-Time Cell Analyzer (RTCA)-DP system.
Results
The results of WB and RT-PCR showed that the expression of four factors in renal cell carcinoma was higher than that in normal renal tissue. And QDB results showed that ANXA4, PYGL, PSMB8 in the vast majority of patients in renal cell carcinoma expression is greater than normal renal tissue, and has statistical significance. At the cellular level, knockdown of PYGL and ANXA4 significantly reduced the proliferation and migration of 786-0 cells.
Key words
renal cell carcinoma ; proteomics; PYGL; QDB; small molecule inhibitor

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A system Approach to Identify Genetic and Environmental Regulators of Virulence in Mycobacterium tuberculosis

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Background

Tuberculosis is caused by Mycobacterium tuberculosis (Mtb) and claims 1.5 million lives annually. Emerging drug resistant strains as well as co-infection with HIV/AIDS increase the depth of the challenge. To date, Mtb research efforts have largely relied on the lab-adapted model strain, H37Rv, and focused on its transcriptional responses. However, clinical isolates of Mtb reveal diverse phenotypic features that are thought to be most directly determined by the state of the proteome. Here, we aim to identify genotype – proteotype relationships resulting in biomolecular networks driving various phenotypes.

Methods

In a pilot stage of the project, we profiled the genome, transcriptome and proteome of six clinical isolates of Mtb from lineage 1 and 2, representing ancient and modern lineages, respectively. In the second phase, we expanded the study to 70 clinical isolates grown under normal and nitric oxide stress by measuring their genome and proteome. We used Illumina NGS to analyze genome and transcriptome of the respective strains and SWATH-MS for the proteomic analyses.

Results

The pilot dataset revealed ~600 significant changes (out of ~2600) at the proteome level including ~40 transcription factors of lineage 2 in respect to lineage 1 strains while their genomic distance is ~1200 SNPs. We elucidated that DosR and IdeR regulon were significantly upregulated in lineage 2 strains while the essential genes remained unchanged. The higher basal expression of DosR, most likely caused through Rv1985c by pknH, explains why lineage 2 frequently encompasses drug resistant strains in comparison to other lineages. Our model indicates transcription factors Rv3133c/DosR, Rv0081 and Rv0576 as playing major role in orchestrating the transcriptional network of lineage 2 in respect to lineage 1 strains.

Conclusions

We have shown that genomic differences between the clinical isolates determine the state of the proteome and mediate different clinically relevant phenotypes.

Keywords

Omics, Tuberculosis

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Detection of Psoriatic Arthritis at Early Onset: A Multi-Proteomic Approach to Developing a Blood Test

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Background: Psoriatic Arthritis (PsA) is a form of seronegative inflammatory arthritis (IA) frequently associated with psoriasis. At early onset, PsA often resembles other disease types - especially rheumatoid arthritis (RA). During the treatment and management of the disease important clinical decisions are made and these have a significant impact on patient outcomes. Making an accurate and early diagnosis is particularly important to ensuring that individual patients receive effective and safe medication. Thus, it is widely acknowledged by physicians and patients alike that a new test is needed to facilitate the early and specific diagnosis of PsA.
Objectives: To (i) identify and verify candidate biomarkers with the potential to segregate patients with PsA from those with RA (ii) explore the value of combining different proteomic discovery platforms.
Methods: Serum samples were obtained from a cohort of 64 patients (32 PsA and 32 RA) defined as early onset (< 12 months) and DMARD naïve. Baseline samples were analysed by nLC-MS/MS (n=64), Luminex xMAP (n=62) and an aptamer based platform called SOMAscan (n=36). A random forest (RF) test was applied to each individual data set as well as to a combined-matched data set (n=36). To verify MS data, a multiple reaction monitoring (MRM) assay was developed 154 LC-MS/MS identified proteins run against the individual patient samples (n=60).
Results: In this study, it was possible to identify and verify proteins with the ability to segregate between patients with PsA and RA. RF analysis revealed the most discriminatory biomarkers from LC-MS/MS Luminex and SOMAscan. Further, RF analyses of MRM data, correctly classified 45/60 patients analysed.
Conclusion: Proteins identified here represent candidates for inclusion into blood based test that could be used in the diagnosis of PsA.
Keywords
Psoriatic Arthritis, Multi-Proteomic Discovery, MRM Based Protein Evaluation.

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The human proteome discovery via diseased testis tissues in search of missing proteins.

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Background
Testis tissues are abundant with protein-coding genes compared to other tissues. Because of this advantage, testis samples are a good candidate to find genes that lack information whether they are protein-coding genes or not. Furthermore, testis related diseases tend to show distinct features of gene expression from one another. In addition, testis diseases compose of high percentage of populations different from normal testis tissues.
Methods
Twenty mg of testis tissues were pulverized and digested using FASP method. Fractionation was processed through ion exchange and reverse phase chromatography based on the STAGE-Tip format. Peptides were analyzed from Q-Executive mass spectrometry coupled with 150 mm C18 columns. Proteome evidences were identified using the Andromeda search engine. Search parameters was peptide FDR < 0.01, protein FDR<0.01 and two or more unique peptides with 9 amino acids.
Results
A large percentage of protein was recovered and was viable to process it through MS/MS spectrometry. The total fraction was analyzed in each testis sample. Protein identification was done on the proteins extracted from three different types of diseased testis tissues. Along comparing proteome profiles of each disease types, missing proteins were observed. We also identified aberrant proteins of nonsynonymous SNP, splicing variants and linc RNA coding peptides. With the profiled data, protein expression levels were analyzed.
Conclusion
From the proteomic analysis of maturation-arrested testis samples, differentially expressed and distinct proteins were identified including missing proteins.

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Development of reference protein extraction protocols to quantify biotherapeutics in mice tissues by UPLC-sMRM

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Quantifying biotherapeutics in tissues is essential to the drug development process. LC-MS/MS is now widely used to quantify proteins in complex matrices. However, to quantitatively recover the proteins from tissues, the sample preparation is crucial. So far, the challenges to obtain an accurate, robust and high-throughput sample preparation for tissues have not been addressed. We describe the optimization of protein extraction and digestion in a wide range of mouse tissues. Five different biotherapeutic formats were chosen: full length IgG, Fab, Fab-PEG, ByBe and a ByBe albumin binder.
Method development is typically performed in control tissues, where only an estimation of the total protein recovery is carried out during optimization steps. To optimise the tissue homogenization, we propose a new strategy where a set of specific endogenous proteins for each tissue by LC-MS/MS is measured. The automated on-pellet digestion was optimized by spiking the five biotherapeutics in tissue homogenates and measuring MS response. Samples were measured by a multiplexed UPLC-sMRM method. The final quantification of the biotherapeutics in tissue was carried out with both a biotherapeutic external standard curve and SIL-peptides as internal standards.
We optimized the protein extraction for mouse liver, kidney, lung, heart, brain, spleen, lymph nodes, intestine and skin. Most of the tissues were homogenised directly in detergents by high focused ultra-sounds. Skin samples were digested by collagenase prior to ultra sound homogenisation. Samples were then digested by an on-pellet procedure using a double LysC/Trypsin digestion. We obtained 100% protein precipitation recovery for the five biotherapeutics despite their different formats and the global reproducibility was <20%.
We have developed robust and automated protocols to extract and digest biotherapeutics in mouse tissues. The method performance meets the guidance for bioanalytical method validation criteria for small molecules. Further work is planned to quantify the five biotherapeutics in in vivo samples.

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Spatial tissue proteomics quantifies inter- and intra-tumoral heterogeneity in hepatocellular carcinoma

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Background

The inter-patient variability of tumor proteomes has been investigated on a large scale but many tumors display also intra-tumoral heterogeneity (ITH) regarding morphological and genetic features. To what extent the local proteome of tumors intrinsically differs remains largely unknown.

Methods

We have developed an optimized protocol for the analysis of Formalin Fixed and Paraffin Embedded (FFPE) diagnostic specimens that is compatible with laser microdissection. We show that our strategy yields reproducible results, allows deep proteome coverage (>6000 proteins), and it is compatible with both Data Independent Acquisition (DIA)- and Tandem Mass Tags (TMT)-based quantitative strategies.

Results

Using hepatocellular carcinoma (HCC) as a model system, we quantified both intra and inter-tumoral heterogeneity across human patient specimen with excellent spatial resolution using biobank FFPE samples. We detected intratumoral variations in the proteome that re-occur across different patient samples and affect clinically relevant proteins. We characterized proteomic features that distinguish neoplastic from the directly adjacent non-neoplastic tissue and derived a molecular signature of HCC by integrating proteomic data from human patient samples and genetically defined mouse models with available gene expression data.

Conclusions

Our work demonstrates the suitability and the benefits of using mass spectrometry based proteomics to analyze diagnostic tumor specimens with high spatial resolution.

Keywords

cancer, heterogeneity, HCC, liver cancer, FFPE, DIA, TMT, personalized medicine, microdissection

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Tear protein analysis in the different phases of wound healing following glaucoma surgery

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Background:
Glaucoma is a chronic neurodegenerative eye disease and the second leading cause of blindness worldwide. The most important form of treatment is the reduction of the intraocular pressure (IOP) by topical medication or surgery. The gold standard of surgical intervention is trabeculectomy, when a channel is generated permitting the flow of aqueous humor. One of the key feature of the surgical success is the proper wound healing which might be impaired leading to excessive scar formation and channel closure. The central goal of the project is to understand the molecular events orchestrating wound healing following glaucoma surgery.
Methods:
Luminex-based 27-plex immunobead assay was applied to study the concentration of 27 cytokine and chemokine molecules on day 0, day 1, day 2 and day 4 after trabeculectomy in the tears of patients. SRM-based targeted proteomics methods were designed to examine the level of growth factors such as IGF-1, HGF, EGF and KGF in tears. The surgical outcome was examined by ophthalmologist by flap grading 1 year after surgery.
Results and Discussion:
Based on the cytokine and growth factor levels observed in different time points we can group patients into three groups: (1) tear protein values are more-or-less constant in all time-points, (2) cytokine levels increase on day 1 and then reduce again, and (3) increased cytokine levels remain high on the following days as well.
Conclusion:
The level of cytokine, chemokine and growth factors examined in the different phases of ocular wound healing along with the results of flap grading can give us more details regarding the understanding of wound healing following glaucoma surgery.

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Efficient and user friendly label-free quantification with the Proline software suite

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Background
Label-free quantification based on precursor ion intensity is a widely used method for quantifying differentially expressed proteins across different conditions or samples. An ideal software solution should allow the production of reliable and comprehensive results, and be flexible enough to allow the integration of existing tools without compromising ease-of-use. Importantly, it should also offer a graphical user interface (GUI) to manually check and fix erroneous quantitative results (bad signal extraction or wrong mapping between identification and quantification data).
Methods
To meet these objectives we developed the Proline software, a next-generation tool based on a modular data processing toolbox. Imported and generated data are persisted in a relational database (PostgreSQL), providing thus a very high sustainability and traceability of the produced results. An original signal detection algorithm was developed taking advantage of the recent mzDB file format. To assess the performance of the label-free module, we used a proteomic standard dataset composed of an equimolar mixture of 48 human proteins (UPS1, Sigma) spiked at different concentrations into a yeast cell lysate background.
Results
Compared to MaxQuant, Proline shows a lower rate of missing values and a better similarity between observed and expected ratios, on the used standard dataset. Moreover it is faster than existing solutions because it leverages the optimized mzDB file format, either for signal processing or raw data display. Visual inspection of the results can be performed through two user-friendly GUIs, Proline Studio (desktop application) and Proline Web (for remote access). Thanks to its architecture Proline may interoperate with any programming language and could be easily plugged into another workflow system.
Conclusions
The benchmarking study shows that Proline provides better label-free quantitative results than its competitors. Proline constitutes thus a very interesting solution combining robustness, performance, modularity and user-friendliness.
Keywords
Label-free quantification; standard dataset; bioinformatics; software; visualization;

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Super-SILAC mix coupled with SIM/AIMS assays for targeted verification of phosphopeptides discovered in hepatocellular carcinoma

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Plentiful studies have established a close association between aberrant phosphorylation and hepatocellular carcinoma (HCC). Here, we applied a quantitative phosphoproteomics platform combining dimethylation labeling and online 3D strong cation exchange chromatography (SCX)-titanium oxide (TiO2)/RP-LTQ-Orbitrap to compare phosphoproteomes between three pairs of HCC tissues and non-tumor counterparts. This analysis yielded 7868 quantifiable phosphopeptides and numerous up- or down-regulated candidates. Increased phosphorylation of LMNA and NIPA was confirmed using specific antibodies. To expand our verification capability, we evaluated the use of LTQ-Orbitrap run in SIM/Accurate inclusion mass screening (AIMS) mode with a super-SILAC mixture as an internal standard to quantify a subset of phosphopeptide candidates in HCC tissue samples. In sample I used for discovery experiment, we successfully quantified 32 (in SIM mode) and 30 (in AIMS mode) phosphopeptides with median coefficients of variation (CVs) of 7.5% and 8.3%, respectively. When the assay was applied to other three pairs of HCC specimens for verification experiment, 40 target phosphopeptides were quantified reliably (~7.5% CV), and more than half of them were differentially expressed between tumor and adjacent non-tumor tissues. Collectively, these results indicate the feasibility of using super-SILAC mix-SIM/AIMS assays for targeted verification of phosphopeptides discovered by large-scale phosphoproteome analyses of HCC specimens.

Key words: Hepatocellular carcinoma; Quantitative phosphoproteomics; SIM/AIMS; Super-SILAC mixture; Targeted verification

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Biomarker Search in Prostate Cancer by Proteomic Approach

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Prostate cancer (PCa) is a non-cutaneous cancer affecting more than two million men in the world. Although Prostate Specific Antigen (PSA), best known biomarker, is associated with significant false positives and this results in up to 700.000 unnecessary prostate biopsies per year. These data highlight the urgent need for additional noninvasive early detection biomarkers for PCa.
Cancer proteome is an exceptionally complex biological sample which contains information from almost all the biological activities that take place in cancer cells, and cancer tissue microenvironment. One major advantage of proteomics is its ability to concurrently check the whole proteome or sub-proteomes such that differentially expressed or modified proteins corresponding to a disease condition can be identified. Methods depending on the type of samples and objective of studies may vary greatly. One of the unique feature of our current study is that, we are using label-free Liquid Chromatography (LC) accompanied with tandem 1D- High Definition Mass Spectrometry (SYNAPT G2-Si with nano lockspray ion from Waters) (LC/MS-MS) to analyze both cancerous and noncancerous prostate tissues and both urine samples. Urine samples desalted by PD-10 columns then degraded by DTT and methylated by iodoacetamyde solution. After trypsinisation step, extracts have been lyophilisated and then dissolved with % 0.1 Formic Acid solution and loaded to the LC MS/MS for both qualified and quantified proteomics analysis. In initial global qualitative analysis, we identified proteins with Protein Lynx Global Server (PLGS 3.0.1). These proteins were clustered with regards to their functions and pathways analysis were investigated for their possible cross talk among other significant oncogenic pathways.Best biomarker candidates will be evaluated by validation methods.
In the present study we analyzed and compared urine of the prostate cancer both healthy volunteers and distinguish the distinct peaks that may refer the potential biomarkers.

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Retinoic acid induced specific changes in the Phosphoproteome of C17.2 Neural Stem Cells

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Background
Protein phosphorylation can regulate most of the important process in stem cells, such as proliferation, differentiation and apoptosis. Retinoic acid （RA）as a clinical drug was used to treat neurological diseases rely on its function of differentiate abduction. But the research of the RA induce C17.2 differentiation remains inadequacy.

Methods
We apply dimethyl labeling combined with the TiO2 phosphopeptide enrichment approach to compare the phosphoproteome of the self-renewal and differentiated cells induced by RA.

Results
We successfully identified 733 and 517 phosphoproteins from the self-renewal and differentiated cells induced by RA respectively. A total of 347 proteins were differentially phosphorylated, and most of which were related to transcription and cell cycle. In addition, we found that PAKs play critical roles in C17.2 cell proliferation. PAK1 activate Wnt/ß-catenin via phosphorylate ß-catenin (p-S654) resulted in expression of c-myc and cyclinD1. Interestingly, Merlin, a cellular substrate of PAK2, functions as a negative growth regulator. Inactive merlin (p-S518) may promote ß-catenin depolymerize from the plasma membrane and localize to cell nucleus.AKT1 can regulate ß-catenin through phosphorylate at S552, which may promote activation of Wnt/ß-catenin pathway.

Conclusions
Overall, our results suggest that protein phosphorylation maybe a key mechanism regulating cell proliferation and differentiation and enhance our understanding of this mechanism.

Keywords: Retinoic acid, NSCs, proliferation, differentiation, phosphoproteome,

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High throughput, single shot plasma proteome profiling on a robust capillary flow setup of 1564 DiOGenes samples.

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Background

Proteins from the blood circulatory system are indicative of the health status of an individual. Comprehensive, robust high-throughput analysis of the proteome will enable holistic analysis of the health state. Recently, large scale studies were presented using nano-flow LC-MS. Though, nano-flow setups are delicate and require long gradient overheads. To significantly reduce this limitation, we established a robust capillary flow LC-MS-DIA.

Method

Plasma samples were prepared with an optimized in solution digestion protocol. Biognosys' iRT kit was spiked into the samples before injection. The samples were acquired with a Waters M-class UPLC connected to a Thermo Scientific Fusion Lumos using EASY transfer line and an EASY spray source. Targeted analysis of DIA runs was performed using Spectronaut.

Results

After testing several combinations, we obtained optimized conditions using a 300 µm inner diameter column (CSH 1.7 µm C18). This setup has the advantage of increased flow rates (5 µl/min) resulting in small injection to injection overhead times (i.e., 5 min) adding to the gradient.
We also performed a gradient length optimization analysis. A gradient of 40 min was finally chosen as an optimal gradient length, resulting in 90% of the maximally achieved identifications. Injection of 5 µg of tryptic peptides resulted in 95% identification (of saturation at 10 µg). Triplicate injections of the same sample resulted in a median CVs at peptide level of 6.8%. Quantitative dataset completeness was 82% at precursor level on a set of 51 measurements (~1.5 days of instrument time).
Then, we carried out a large-scale plasma sample study, comprising 1500 samples of the Diet, Obesity and Genes (DiOGenes) project of the FP7, which were acquired with the above presented capillary flow setup.

In conclusion, we developed a simple and robust workflow for label-free proteome quantification with high quantitative precision and high reproducibility.

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Proteomic Cinderella: subtle analysis of bulky MS/MS data in one night

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Background
Proteomic challenges, stirred up by the advent of high-throughput technologies, produce large amount of MS data. Nowadays even a small biology lab equipped with the state-of-art instruments can become a big-data source. The simplicity of the advanced analysis is overlapped by far with the complexity of interpretation and storage of the information obtained. Routine manual search does not satisfy the “speed” of modern science any longer.
Methods
In our work the necessity of single-thread analysis of bulky data emerged during interpretation of HepG2 proteome profiling results. We searched for proteoforms in 2DE gel separated into 96 cells and analyzed obtained MS/MS data (192 raw files of total volume 114 Gb) over and over again – with variable settings, databases, and combinations of search engines.Effective solution for customized search strategy, realized by M.Vaudel et al in open-source graphical user interface SearchGUI (http://searchgui.googlecode.com), allowed us to solve this rather sophisticated task. We compared contribution of each of seven search engines (X!Tandem, MS-GF+, MSAmanda, MyriMatch, Comet, Tide, Andromeda, and OMSSA) into total result of proteoforms identification and optimized set of engines working simultaneously. We also compared the results of our search combination with Mascot results using protein kit UPS-2, containing 48 human proteins.
Results
We selected combination of X!Tandem, MS-GF+ and OMMSA as the most time-efficient and productive search combination. We added homemade java-script to automatize pipeline from files’ picking to reports’ generation. These settings resulted in rise of the efficiency of pipeline unobtainable by manual scouting: the analysis of 192 files searched against up-to-date configuration of human proteome (42153 entries) downloaded from UniProt took 11 hours.
Conclusions
Like Cinderella, separating the wheat from the chaff in one night, integration of search algorithms tailored for certain scientific need can rapidly translate massive data into biological knowledge.
Keywords
Search Algorithms, MS/MS, Proteoforms, Proteomics

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A multiplexed LC-MS/MS based assay for quantification of biomarkers of neurodegeneration in mouse brain homogenates

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Background:
Quantifying biomarkers forms an important part of a drug discovery programme and is increasingly used to support characterization of preclinical in vivo models of disease in rodents. Here we describe a multiplexed LC-MS/MS based assay for quantification of 6 biomarkers of neurodegeneration: Synapsin 1, PSD-95, VGLUT1, Neurofilament light chain, SV2C and Synaptophysin in mouse brain.

Method:
The sample preparation involved homogenisation of the brain samples in the presence of surfactant followed by surfactant aided on-pellet digestion using trypsin. Post digestion, the samples were concentrated using solid phase extraction. In silico digests of the protein sequences were performed to determine theoretical tryptic peptides. Peptide libraries were synthesised based on these in silico digest and 3 best responding peptides per protein were chosen based on the mass spectral and retention time similarity between peptides present in brain homogenate to that of peptide library. Quantification of the endogenous proteins was carried out using typical AQUA peptide workflow.

Results:
Four different surfactants were assessed for precipitation: RIPA, SDS, Rapigest and Sodium deoxycholate; based on the MS response of the peptides, RIPA buffer proved to be better in extracting the proteins compared to other surfactants. Using the current methodology, the levels of low and high abundant proteins were measured at 49 and 2085 ng/mg total protein respectively, and the assay reproducibility was <30% for digestion replicates and <10% for injection replicates.

Conclusion:
The results above suggest this method can be used to measure levels of endogenous markers in whole brain homogenates. This approach will be used to measure the levels of the above mentioned biomarkers in in vivo models of neurodegeneration to characterise the models and to understand the efficacy of biotherapeutics tested in these models.

Keywords:
Biomarkers, Neurodegeneration, Quantification, LC-MS/MS

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Optimisation of sensitive and robust methods for characterisation of the PLK4-regulated phosphoproteome

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Background: Mass spectrometry-based methods are increasingly being used to study cellular phosphorylation events, with ever increasing numbers of phosphopeptides being reported. However, confidence of phosphosite localisation is often not paid sufficient attention. Here, we exploited the dynamic capabilities of the Orbitrap Fusion Tribrid mass spectrometer, to optimise a phosphoproteomics workflow in which phosphopeptides are identified both in high numbers and with confident phosphosite localisation. Robust phosphoproteomics workflows are essential to interrogate cell cycle-mediated changes in cellular phosphorylation status, such as those regulated by the centriole localised polo-like kinase 4 (PLK4).
Methods: A library of phosphopeptide standards and phosphopeptides enriched from a U2OS cell lysate were analysed by LC-MS/MS on an Orbitrap Fusion using seven different acquisition methods, evaluating the type and rules for triggering fragmentation (HCD, EThcD, ETcaD; observation of neutral loss (NL)) and the mode of MS2 analysis (orbitrap (OT), or ion trap (IT)). Data were processed in Proteome Discoverer (MASCOT & ptmRS) and MaxQuant (Andromeda and PTM-score). Phosphopeptide identifications and site localisation scores were assessed. The optimized methods were subsequently used to investigate the PLK4 regulated phosphoproteome in transfected U2OS cells.
Results: Of the seven MS methods tested, HCD-OT provided the highest number of phosphopeptide identifications with ~70% of phosphosites assigned with high confidence. EThcD-IT provided the fewest number of phosphopeptides but with the highest percentage of confidently assigned phosphosites (~80%). Combining HCD OT with NL-triggered EThcD IT proved to be a promising method, generating high numbers of phosphopeptides with ~75% of all phosphosites assigned with high confidence. Application of our optimised phosphoproteomics workflow is being used to reveal patterns in PLK4-mediated cellular phosphorylation events.
Conclusions: Systematic evaluation of MS methods on the Orbitrap Fusion mass spectrometer, has allowed us to define a method that balances throughput of phosphopeptide identification with robust phosphosite localisation, essential for biological characterisation.

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Improved detection sensitivity of quantitative changes for low-abundant proteins by data curation of PLGEM-STN p-values

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Background

In the label-free quantitative proteome analysis, PLGEM-STN statistical analysis for shotgun proteomics data has been widely employed for the detection of relative protein abundance differences. However, we observed that PLGEM-STN p-values (≥0.01) are biased more toward proteins with high-abundance than low-abundance. To rescue numbers of proteins with low expression, we evaluated quantitative changes of proteins have p-values within 0.01 ≤p-value ≤0.05 and with at least 5 spectral count differences between the comparing sample sets and identified significant numbers of differentially expressed proteins (DEPs) between MDA-MB453 breast cancer cells grown under normal and glucose-deprivation (DG) conditions.

Methods

Total proteins were extracted from MDA-MB453 breast cancer cells grown under normal and DG conditions. Following the in-solution digestion, peptides were analyzed in triplicate using a Thermo Scientific Q-Exactive MS. The LC-MS/MS data were automatically processed using the SEQUEST-SORCERER protein database search platform. Relative protein quantitation was accomplished spectral counting software. The normalized spectral counts from triplicate datasets were compared using the PLGEM software in order to identify DEPs in MDA-MB453 grown under DG condition.

Results

In total, 2,525 proteins were identified. Of them, 681 proteins (p-value <0.01) were identified as DEPs in MDA-MD453 grown under DG condition. We further analyzed proteins have p-values within 0.01 ≤p-value ≤0.05 and with at least 5 spectral count differences and identified 411 proteins altered in response to the DG condition. Network analysis and western blot analysis were performed on a subset of proteins (UBE2H, UBE2D3, UBE2N, UCHL5, and PSMD) to validate the observed abundance differences.

Conclusions

This study demonstrates assessment of quantitative changes of low-abundant proteins by extended curation of PLGEM-STN p-value scores to increase the number of DEPs in the shotgun proteome analysis of complex biological samples.

Keywords

Quantitative proteome, Spectral counting, Breast cancer

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Bringing proteomics data analysis into the cloud

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Background
With faster mass spectrometry (MS) instrumentation in ever higher resolution, the amount of available proteomics datasets is approaching big-data proportions. This faces data analysis with the challenge of scalability. High Performance Computing (HPC) environments are only selectively available to proteomics research groups and come with the added complexity of deploying an analysis workflow to the local e-infrastructure and a limited feasibility to later share this workflow with the research community. Increased cloud computing availability in large research programmes offers the possibility to bring the analysis closer to the data and achieve truly scalable data analysis.

Methods
Here, we present an integration of established proteomics tools into a flexibly deployable system that plugs into the EMBL-EBI "Embassy Cloud, as a proof of concept. We containerised over 100 tools for proteomics analysis, centred around the OpenMS framework, to achieve the necessary portability for cloud application. The system allows to build new workflows without programming or having an intricate knowledge of the underlying e-infrastructure. This resource was built on top of the Galaxy/Kubernetes integration produced within the Phenome and Metabolome aNalysis project (PhenoMeNal - http://portal.phenomenal-h2020.eu) and adds the capability of advanced across-omics applications.

Results
We provide the availability of custom and pre-built proteomics analysis workflows for the cloud based on the OpenMS framework. Our system can be deployed via Kubernetes in all major cloud environments. Established workflows are available for PRIDE data within the EMBL-EBI "Embassy cloud".

Conclusions
The deployment of proteomics analysis workflows in the EMBL-EBI "Embassy cloud" shows the applicability of cloud infrastructures within proteomics research and underpins the potential to truly scale up data analysis. The option of federated cloud based cluster systems allows the development of high availability, high scaling analysis systems.

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Identification of citrullinated proteins in human tissues

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Background
Citrullination is an arginine PTM catalyzed by peptidylarginine deiminases. It only leads to the increase of 0.98 Da in mass, but the loss of a positive charge may cause structural and/or functional alterations of modified proteins. Citrullinated proteins are related to several diseases e.g. rheumatoid arthritis, cancer, neurological diseases, but the physiological role of citrullination remains unclear, in particular because of the limitations in identifying citrullination on proteins and pinpointing the sites in vivo.
Methods
The human proteome datasets (1,116 raw files) of 31 deep proteome were measured by Thermo Orbitrap QE in our lab (Wang et al., unpublished data). The files were processed and searched with Mascot considering deamidation on N/Q and deamidation on R (citrullination) as variable modifications. The fragment neutral loss (-43 Da) of citrullination was considered as scoring fragments in Mascot. The DAT files were loaded into scaffold for manually spectrum inspection. The resulting candidate citrullination peptides and their permutations were synthesized (JPT Peptide Technologies, Germany) and analyzed on an Orbitrap Fusion Lumos using multiple fragmentation types.
Results
After database search, ~2600 spectra were manually inspected with an emphasis on the overall spectrum quality, modified fragment ions, and the corresponding neutral loss ions. The discrimination of N/Q deamidation and R citrullination required the comparison and alignment of candidate peptide-spectrum-matches with a citrullination-specific spectral library consisting of ~4000 synthetic peptides. Using the described strategy, we identified known and several novel citrullinated proteins and could pinpoint their sites.
Conclusions
The analysis of protein citrullination requires a reliable multi-pronged approach to maximize the number of candidates and to eliminate false-positive results. This study represents the first high quality human in vivo “citrullinome” based on thorough manual spectrum interpretation and a citrullination-specific spectral library. The spectral library will be available on ProteomeTools (http://www.proteometools.org).
Keywords
citrullination, spectral library, human proteome

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EFFECTS OF MYRISTIC ACID ON METABOLISM IN HEPG2 CELLS INVESTIGATED BY A PROTEOMIC APPROACH

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Background
Myristic acid (C14:0) is a saturated fatty acid occurring in animal and vegetable fats. Previous studies suggested that C14:0 may influence plasma lipids and modulate the risk of some metabolic disorders, like non-alcoholic steatohepatitis. The aim of the present study was to detect specific protein patterns related to C14:0 exposure, shedding light on the effects of C14:0 on HepG2 cells metabolism. The effects of C14:0 were then compared with the modulations on the HepG2 proteome induced by palmitic acid (C16:0) and oleic acid (C18:1).

Methods
HegG2 cells were exposed for 24h to C14:0, C16:0 and C18:1, ranging from 50 □M to 500 □M. After cell lysis and proteins extraction, whole proteomes were analyzed using nanoHPLC -maXis II ETD mass spectrometer. Enrichment analyses were performed using Reactome and String databases.

Results
MS analysis revealed that C14:0 modulated a total of 93 proteins in HepG2 cells, of which 15 involved in lipid metabolism. C 14:0 modulated more proteins than the saturated fatty acid C 18:1 (85) , but less proteins compared to the cytotoxic C 16:0 (447). Among the most enriched pathways specifically deregulated only by C14:0 it was possible to identify protein folding, protein complex biogenesis and assembly, as well as vesicle-mediated transport and exocytosis. Accordingly, among the most modulated proteins ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), small glutamine-rich tetratricopeptide repeat-containing protein alpha (SGTA) and vesicle-associated membrane protein-associated protein B/C (VABP) were found. Interestingly it has been reported that VABP is involved in the Unfolded Protein Response (UPR) following ER stress conditions.

Conclusion
This study provides for the first time an overview of protein patterns specifically related to C14:0 exposure in HepG2 cells. Further functional validations may provide robust data to assess the role of this fatty acid in cardiovascular disease.

Keywords: myristic acid, fatty acids, cardiovascular disease, proteomics.

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Regional variation and novel pathogenesis in the human Alzheimer's disease brain

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Background. Alzheimer's disease (AD) is a leading cause of death in the developed world. Despite increased focus, there are no effective therapies; even the root causes of AD remain controversial. While amyloid plaques and tau tangles are thought key to pathogenesis, targeting these pathways has yielded little success. New targets, and new insights into disease development, are urgently required. We have analysed protein expression across six distinct regions of human AD-affected brains versus age-matched controls to reveal novel cellular mechanisms associated with disease.

Methods. Matched brain tissue (n=9 per class) was acquired from the Auckland brain bank and six regions covering affected and 'spared' regions, namely hippocampus (HP), entorhinal cortex (ENT), cingulate gyrus (CG), sensory cortex (SCx), motor cortex (MCx), and cerebellum (CB) were dissected. Relative protein expression was determined by iTRAQ LC-MS/MS analysis followed by Bayesian statistics to determine differences between cases and controls. Pathway analysis (Ingenuity) and correlation network analysis (Moduland/Cytoscape) were subsequently performed to compare both disease vs. control and distinct brain regions.

Results. Over 5000 proteins were quantified in total, with 1,903 quantified by ≥3 peptides in all six regions. Pathway analysis revealed neuroinflammation across the brain, albeit more marked in affected regions. Additional metabolic and signalling pathways were observed in more affected regions, in agreement with previous studies and suggestive of an 'evolution' through the organ. Strikingly, cerebellum, a region thought to be spared, exhibited a significant number of alterations in its proteome which were unique, and which could be protective against neurodegeneration in this region.

Conclusions. We have developed an in-depth picture of protein expression in late-stage AD, with some regions displaying what we believe to be an 'early-AD' phenotype, and an active protective phenotype in cerebellum. This provides critical new data on AD pathogenesis, and reveals new pathways for potential therapeutic targeting.

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Alterations to HDL protein composition in abdominal aortic aneurysm patients

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Background Abdominal aortic aneurysm (AAA) is a permanent dilation of the aorta resulting from weakening of the arterial wall. While low high-density lipoprotein (HDL) cholesterol levels have been consistently associated with human AAA and treatment with HDL is able to decrease experimental AAA formation, the underlying mechanism has not been fully elucidate yet. Since HDL is a transport platform of constitutive and non-permanently associated plasma proteins and its potential vasculoprotective effects have been associated with its composition, we aimed to analyze the proteome of HDL from AAA patients.

Methods We performed multiplexed, quantitative high-throughput proteomics analysis of: i) HDL obtained from plasma (controls and AAA patients, n=10 each) and ii) HDL isolated from plasma of healthy volunteers and incubated ex vivo with tissue-conditioned media (healthy wall, AAA wall and AAA thrombus, n=4 each). Samples were fractionated by cation exchange and subjected to LC-MS/MS analysis. Statistical analysis was performed based on the weighted spectrum, peptide and protein and systems biology triangle models to pinpoint significant changes in protein abundance and function.

Results HDL isolated from plasma of AAA patients showed elevated abundance of several proteins, including the antioxidant paraoxonase/arylesterase 1 (PON1), whereas the proinflammatory C4b-binding protein was decreased and the constitutive apolipoprotein A1 (ApoA1) remained unaltered. In contrast, upon incubation with AAA tissue-conditioned medium, HDL showed strong downregulation of PON-1 and ApoA1 and increased C4b-binding protein with concomitant increase of reversible thiol protein oxidation. The main pathways associated with HDL alterations in AAA were oxidative stress and immune-inflammatory responses.

Conclusions HDL protein composition is markedly altered in AAA patients compared to controls, showing differences between circulating and tissue-like HDL. This study further supports the concept of tissue dissociation of important vasculoprotective HDL proteins under proinflammatory and prooxidant conditions as those present in AAA.

Keywords Abdominal aortic aneurysm, HDL, plasma, thrombus-conditioned medium

Oxidized methionine levels in serum proteins as potential clinical biomarkers to assess oxidative stress status

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Background
Oxidative stress is believed to play important pathophysiological roles in the development of major human diseases. However, no conventional techniques can accurately detect oxidative stress status in human biofluids.

Methods
Two µL of serum samples collected from healthy volunteers and patients with type 2 diabetes with and without renal failure were reductively alkylated, completely digested with trypsin and Lys-C using phase transfer surfactant, and subjected to highly sensitive analysis using nano-flow LC-MS/MS. Fifty-three identified peptides containing Met residue were quantified using LTQ-Orbitrap Discoverer equipped with conventional HPLC. Signal intensities of peptides were quantified using extracted ion chromatograms (XIC) of LC-MS analyses with mass tolerance window of 6 ppm. Ratio of XIC of peptides containing oxidized methionine residue to that of unoxidized methionine ([Met(O)]/[Met]) were determined.

Results
Four tryptic peptides containing albumin (Met-111), albumin (Met-147), immunoglobulin (Ig)γ1 chain C region (Met-135) or α1-antitrypsin (Met-409) were clearly separable from other peptides and had sufficient mass spectrum intensity with high reproducibility and without detectable mis-cleavage products. Their [Met(O)]/[Met] values were neither affected by clotting time at room temperature for serum separation, nor by serum handling procedures, such as reductive alkylation, trypsin digestion time, repeated freeze-thaw. [Met(O)]/[Met] value in serum albumin (Met-111) and (Met-147) showed significantly high values in patients with diabetes and renal failure and than in healthy controls and in healthy smokers than in non-smokers.

Conclusions
[Met(O)]/[Met] levels were reproducibly determined using human serum samples obtained at clinical laboratory. [Met(O)]/[Met] values in serum albumin (Met-111) and (Met-147) were elevated in diseases and conditions inducing oxidative stress. This mass spectrometry approach to quantify Met oxidation in serum proteins could be applied to identify other Met-containing serum proteins as biomarkers for human diseases.

Keywords
serum, protein, oxidative stress, methionine sulfoxide

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Cytoskeleton and nuclear lamina affection in Osteogenesis imperfecta: a functional proteomics perspective.

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Background: The brittle bone disease osteogenesis imperfecta (OI) is a heterogeneous collagen-related disorder with dominant, recessive and X-linked transmission, which is mainly caused by mutations in collagen I genes or in genes involved in collagen I metabolism.
There is no definitive cure for OI and, in order to identify targets for treatment development, we performed a functional proteomic study to delineate affected molecular pathways in primary fibroblasts from dominant and recessive OI patients, whose phenotypes ranged from moderate to lethal.
Methods: OI cells were analyzed applying 2-DE/MALDI-TOF MS, Western blotting and confocal-immunofluorescence. Dominant fibroblasts were also investigated by a TMT-labeling/Nano-LC-MS/MS quantitative approach. Data functional processing was achieved by MetaCore resource.
Results: Protein expression profile differences, identified among OI forms and controls and among dominant OI dead patients and survivors, were cross-linked in MetaCore networks. These latter pointed to protein differences as intersecting factors among cellular pathways involved in cytoskeleton and nuclear lamina organization, in cell signaling and protein trafficking, as well as in dentinogenesis and osteogenesis. Nuclear lamina and/or cytoskeleton structural impairments were then proved, by immunofluorescence, in patients fibroblasts.
Noteworthy, different “capability” in handling cytoskeleton affections emerged to influence/determine survival of dominant OI patients.
Conclusions: Despite the majority of deregulated proteins identified in dominant and recessive OI forms are different, specific biological processes and molecular functions are characteristically affected in OI, independently of Mendelian mode of transmission and of mutated gene. Both dominant and recessive OI present intracellular-structural-protein aberrances and we pointed them as targets for OI treatment. Deregulated pathways and molecular aberrances, here delineated, are similarly reported in other rare genetic disorders, thus suggesting the possibility to define common pharmacological targets to support conventional approaches in treating different disorders.
Keywords: cytoskeleton; nuclear lamina; signal-transduction.

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Label free quantitation: comparative analysis of XIC and SpCs based methods

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Background
New label-free Differential Proteomics approaches focus on performing relative quantification of proteins between different samples without labels, significantly reducing costs and time of analyses. For this purpose, they rely on comparing intensities of extracted ion chromatograms of peptides attributed to the protein, (XIC methods), or their spectral counts, (SpCs methods). With this work, we compared these methods and defined new complexity-based normalization approaches to be applied for SpCs quantification.

Methods
We compared XIC and SpCs strategies analyzing standard protein mixtures with increasing complexity to evaluate and optimize the parameters for protein identification and quantification, introducing strict criteria. At the same time, we tested several complexity-based normalization approaches for SpCs. LC-MS/MS data were acquired on an Orbitrap XL and the bioinformatic workflow was executed in MaxQuant. Optimized parameters were later employed in a study concerning Huntington's Disease. Three total protein extracts from cerebral cortices of wild type mice and three from Huntington's Disease mice model zQ175 were separated by SDS-PAGE. Each lane was fractionated in 29 slices that were in situ hydrolyzed, obtaining peptide mixtures successively analyzed by LC-MS/MS.

Results
Raw data were processed in MaxQuant according to the parameters early optimized, while statistical analysis was performed in Multiexperiment Viewer. Methodological comparison of XIC and SpCs approaches on standard proteins proofed LFQ (MaxQuant XIC method) and complexity-based SpCs approaches to be reliable, so quantitative analyses were carried out on real samples for both, comparing their results. The 75 common proteins were chosen for further functional analyses.

Conclusions
The functional analyses were performed separately on upregulated and downregulated significant proteins, revealing alterations already known in literature. This demonstrates both correctness of our strict criteria for proteins identification and quantification and the importance of accounting for sample complexity in SpCs normalization approaches.

Keywords
Label-free Quantification; MaxQuant; Huntington Disease

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Monitoring oxidative stress and progression to cell death: are we closer to Parkinson's disease diagnosis?

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Oxidative stress is perhaps the most common factor and the main cause of cell death involved in several disorders. It may occur through diverse mechanisms leading to different responses, which can be reflected in the secreted molecules. Under controlled conditions, these responses can be reflected by the molecules secreted by cells, therefore the secretomes can be an important source of potential biomarkers, more likely to be reflected in biofluids.
The aim of this work was to obtain a panel of markers from the secretome analysis which can be used to distinguish the changes caused by oxidative stress regulation or induction of cell death, and be able to monitor the indicators of cell death progression in blood.
A cell model was treated with different stimulus to induce oxidative stress with and without cell death. Their secretomes were analyzed by SWATH-MS and the markers identified were validated in cerebrospinal fluid and serum from animals from a model of Parkinson's disease.
A large number of molecules were quantified between control and mild oxidative stress conditions. Four groups of molecules were highlighted by allowing a clear distinction of the conditions, and can be considered indicators of stress previous to cell death. In addition, 24 proteins markedly increased in the cell death condition were considered good extracellular indicators of cell death. From these, three reveal to be able to distinguish the 6-OHDA injected animals from the controls in both CSF and serum samples.
In summary, an integrative approach was introduced, and successfully applied to identify oxidative stress biomarkers in the secretome. To transpose the proposed method to clinical diagnosis, these potential biomarkers were further validated in CSF and plasma from an animal model subjected to an oxidative stress insult, revealing to be capable to distinguish the 2 groups with or without neurodegeneration.

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A high-throughput quantitatively platform for serum stathmin detection in esophageal cancer

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Background Esophageal cancer (EC) is one of the most common malignant cancer worldwide and most patients died of recurrence and metastasis. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype in Asia. Therefore, determining factors correlated with metastasis and motility has important clinical significance.
Methods ESCC tissues with lymph node metastasis (n=4) or without metastasis (n=4) and their adjacent epithelia were analyzed via 2-DE-MALDI-TOF-MS strategy, and stathmin, one of the differentially expressed proteins, was further validated. Anti-stathmin monoclonal antibodies targeting a unique peptide epitope, a sandwich ELISA assay and a competitive inhibition AlphaLISA platform for serum stathmin detection were generated.
Results Compared with healthy individuals, serum stathmin levels in ESCC patients were found increased by ELISA assay (n=153). Subsequently stathmin was identified from ESCC cell exosome and suggested that it may be secreted into peripheral blood by exocrine secretion. Underwent large number validation of clinical samples (n=645) by AlphaLISA assay, serum stathmin can be used as a specific marker of ESCC with 92.9% and 81.5% of specificity and sensitivity (AUC= 0.928).
Conclusions We established a high-throughput quantitatively serum stathmin detection platform with high sensitivity and specificity. Stathmin may be used as an important serum marker to assist ESCC diagnosis and therapeutic monitoring.

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A user-friendly spectrum clustering software for optimised proteomics data analysis

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Background

In 2016 we launched our PRIDE Cluster resource (http://www.ebi.ac.uk/pride/cluster/), where we clustered ~190 million spectra coming from public "complete" datasets available in the PRIDE Archive repository, using the spectra-cluster algorithm. We identified originally unidentified spectra by grouping them with identified ones. Additionally, we found millions of high-quality spectra coming from hundreds of datasets that were clustered together but were all unidentified. Our next goal is to apply the same approach to individual datasets. However, standalone software able to analyse results coming from spectrum clustering approaches is currently not available.

Methods

Here we present our newly released spectra-cluster toolsuite (https://spectra-cluster.github.io). The toolsuite has three major components: 1) The spectra-cluster API that supports the development of MS/MS clustering algorithms; 2) multiple implementations (command line version, graphical user interface, Hadoop version, and a ProteomeDiscoverer node) of the spectra-cluster algorithm; and 3) a collection of tools to analyse and process the clustering results.

Results

We used the spectra-cluster toolsuite in a label-free quantitation workflow where clustering was used as alternative to the commonly found "match-between-runs" feature and show that it considerably improved the quality of the label-free quantitation results. Next, we analysed 60 human foreskin samples that are known to be densely colonised by a high number of bacteria. Originally, only 20% of the total spectra could be identified. We used the spectra-cluster algorithm to extract spectra observed in many samples, but that remained unidentified. We processed them using de novo sequencing and open modification searches, which allowed us to characterise the species and PTMs present. Additionally, using the clustering results we could characterise the samples independently of the identification data.

Conclusions

Spectrum clustering can be used to optimise proteomics data analysis. The spectra-cluster toolsuite ProteomeDiscoverer node as well as the spectra-cluster-py analysis framework enable a simple integration into existing popular workflows.

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Dessign of a targeted proteomics assay for the diagnosis of invasive candidiasis.

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Background
Candida albicans is an opportunistic pathogenic fungus that can be part of the human microbiota but can also cause infections, as systemic candidiasis that produces high morbidity and mortality in immunocompromised subjects. An accurate diagnosis of invasive candidiasis is frequently only possible at an advanced stage leading to delays in appropriate antifungal therapy. Thus, it is necessary to identify new diagnostic biomarkers and the development of new strategies for early diagnosis of the invasive candidiasis.

Methods
C. albicans SC5714 strain was used. Mice were infected and the blood was collected at ten days. Multiple Reacting Monitoring (MRM) methods have been developed using Skyline and tested on QTRAP 5500 mass spectrometer. In all the analyzed samples, abundant human and mice plasma proteins were removed by immunodepletion.

Results
In order to select C. albicans proteins that have potential as biomarkers for diagnosis and monitoring of invasive candidiasis, we have used our previous proteomics studies to characterize the extracellular medium of C. albicans. We searched more abundant proteins and characteristic of the C. albicans. MRM analyses were performed on different samples: secretome from C. albicans under hypha-induced conditions (incubated with 1% human serum) as control sample, plasma from C. albicans incubated in human blood, and the sera from the blood of C. albicans infected mice.
MRM assays were designed to detect 31 proteotypic peptides belonging to 12 proteins, using as internal standard, synthetic stable isotope-labeled peptides. We were able to detect six and one peptides in human blood and the murine model respectively.

Conclusions
Targeted proteomics was useful to detected peptides in serum from an in vivo model of systemic candidiasis. The improvement of the method would be useful to develop novel approaches for the diagnosis of invasive candidiasis

Keywords
MRM, Candidiasis, biomarkers diagnosis.

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Proteomic Approaches to Identify the Mechanism of rapid progression of Alzheimer's Disease

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Recent reports demonstrating one-third cases of Alzheimer’s disease progressing very rapidly, mimicking prion-based Creutzfeldt-Jakob disease (CJD) and are misdiagnosed. Altered cerebrospinal fluid biomarkers and neuropathology features give some indications, however, still, there has been no quantitative study depicting risk factor contributing the fast progression and rapid decline of cognition in AD.
In combination with affinity enrichment and high-resolution label-free Q-TOF LC-MS/MS analysis, we quantitatively analyzed globe wide proteome alteration in thirty cortical brain samples with rapid (rpAD) and slow progressive AD (spAD). A conservative approach of selecting only the consensus results of four normalization methods was suggested and used. Furthermore, we verified differentially expressed proteins at transcriptional and translational level.
A total of 79 proteins were shown to be significantly differentially abundant (p-values<0.05, corrected for the multiplicity of testing) in rpAD and spAD versus control brain samples (Ctrl). Forty-eight proteins were specifically showed different levels specifically in rpAD subjects. Interestingly, in our rpAD dataset selectively, we identified an altered expression level of proteins involved in the metabolism of glucose leading to disrupted ATP energy production.
We substantiate that the aberrant metabolic networks are a specific phenotype of the brain with the rapid decline and fast progression of AD.

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comprehensive bioinformatics workflow for analysis and identification of human missing proteins

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Background
Missing proteins are predicted proteins from genomic or tran-scriptomic data, and still lack reliable expression evidence. According to the last release of nextprot database (2017-01-23), Human proteins are classified into 17008 identified (PE1), 2,579 missing (PE2-4) and 572 uncertain (PE5). We approached to analyze the physicochemical properties of human proteome and then searching the unique tryptic peptides of these missing and uncertain proteins (>= 9 aa) in peptide lists of PA, GPM and PRIDE cluster databases.
Results
Analysis of physicochemical properties of human proteome showed that the average MW of identified (PE1), missing (PE2-4) and uncertain (PE5) proteins was 66.6KD, 43.8 KD and 28.5 KD, respectively. Also, the frequency of identified proteins, which have more than 3 isoforms, is 4- and 15-times higher compared to the missing and uncertain proteins, respectively.
In addition to, 580, 1840 and 19 unique peptides of missing and uncertain proteins matched to PA, GPM and PRIDE cluster peptide lists, respectively. These matched peptides correspond to 336 missing and uncertain proteins by more than one unique peptides (>= 9 aa) and 402 proteins by only one unique peptide (>= 9 aa).

Conclusion
Our approach showed that missing and uncertain proteins tend to generate less number of unique tryptic peptide due to limitations in length and number of isoforms. Wide screening of different public peptide databases demonstrated that more than 10% of the missing and uncertain proteins confidently identified by more than one unique peptide. In addition to, 13% of them identified by only one unique peptide.

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Oncogenic protein network reconstruction using multi-omics identifies the ELF3 tumor suppressor as a Wnt antagonist

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Background

The canonical Wnt signalling pathway regulates fundamental cellular processes and is frequently dys-regulated in colorectal and many other cancers. Beta-catenin is a key downstream effector of canonical Wnt signalling, and oncogenic mutations of Beta-catenin result in stabilization and accumulation of the protein resulting in widespread alteration of molecular networks at multiple levels of organisation.

Methods

To understand how oncogenic β-catenin mutations transform molecular networks in cancer cells, we used a multi-omics approach. Using interaction proteomics (Affinity-Purification Mass-Spectrometry), quantitative LC-MS/MS and transcriptomics (RNA-Seq), protein-protein interactions, protein expression and gene-expression was profiled in isogenic cells uniquely expressing either the oncogenic mutant Beta-catenin or wild-type Beta-catenin and integrated mutant and wild-type protein networks constructed.

Results and Conclusions

Comparison of the interaction proteome, expression proteome and transcriptome datasets with respect to enriched biological pathways and processes showed significant overlap between the datasets, indicating that the Beta-catenin mutation induces a coherent cellular response across the interaction proteome, quantitative proteome and the transcriptome. Although similar processes and pathways were invoked, we found that distinct differences in the topology of networks constructed with the different omics data-types. Network edge types between proteomic and transcriptomic datasets were found to be highly complementary, showing that an integrated multi-omics approach to network characterization is able to more comprehensively re-construct the underlying network that individual omics data-types. Finally we identified a strong signature of epithelial-to-mesenchymal transition (EMT) associated with mutant Beta-catenin. Computational network analyses identified the epithelial-specific transcription factor, ELF3, as a repressor of EMT, and we showed that ELF3 is an antagonist of Wnt/Beta-catenin signalling, consistent with its role as a tumour suppressor. In summary, integrated, multi-omics analyses allows for comprehensive characterization of oncogenic signalling networks and the identification of network nodes with key regulatory roles.

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Proteomics reveals associations between protein abundance and sepsis phenotypes

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Background

Proteomic analyses are intensely used to search for biomarkers for diagnosis or prognosis of therapy. This requires the availability of extensive well characterized sample collections as well as the ability for reproducible quantitation of biomolecules in sufficient sample series. Proteomics screens of large sepsis cohorts are still limited and thus in this study, human plasma proteomics was applied to screen for protein signatures improving the characterization of sepsis and SIRS patients.

Methods

We analyzed depleted plasma samples of 428 patients collected 24 and 48 hours after an intensive care unit admission. Based on clinical parameters patients were categorized into systemic inflammatory syndrome (SIRS), severe sepsis and septic shock. Samples were subjected to liquid chromatography-mass spectrometry based bottom-up proteomics.

Results

In clinical practice the SOFA score (Sequential Organ Failure Assessment) is used to predict the in-hospital mortality of severe sepsis. In a proteomic analysis of 101 plasma samples of sepsis patients we discovered a large number of proteins which correlated to SOFA. Further characterization of the plasma proteome across patient samples resulted in a specific protein signature that described differences between SIRS and septic shock patients. Results of proteome analyses were validated in a second sample set including 327 patients. Based on the two complex data sets a classification via random forest was used to discriminate sepsis and SIRS.

Conclusion

Application of gel-free proteomic analysis as a high-throughput technology enabled the identification of sepsis specific protein signatures. After proteomic analysis from a well characterized patient cohort, which were assigned to different disease stages by clinical data, proteins with association to sepsis and sepsis-related parameters were identified. The sepsis specific protein signatures could be validated in a second larger data set. Patient with and without prior infection could be distinguished based on proteomic data.

Keywords
sepsis, SIRS, clinical proteomics, biomarker

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New targets for inhibition of the PDL1 – PD1 immune checkpoint

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Background
Cancer cells exploit the expression of the programmed death-1 (PD-1) ligand 1 (PD-L1) to elude T-cell mediated immunosurveillance. Consequently, therapies that restrain PD-L1 mediated tumour tolerance have shown significant clinical success, highlighting the urgent need to understand the molecular regulation of PD-L1 expression.

Methods
Genome wide CRISPR/Cas9 forward genetic screen for determinants of PD-L1 cell surface expression. Quantitative proteomic analysis of the plasma membrane using isobaric tag technology. Flow cytometry. Immunofluoresecence microscopy. IL-2 and TNFalpha secretion assays as a measurement of T-cell activation.

Results
A genome wide CRISPR/Cas9 forward genetic screen identified “PDL1-Associated Protein (PDL1-AP)” as a novel factor impacting the level of cell surface PD-L1. PDL1-AP associates with PD-L1 and is required for cell surface stability but not trafficking. PD-L1 is also associated with PDL1-AP in recycling endosomes and in the absence of PDL1-AP is degraded in a lysosome-dependent manner. To determine additional proteins whose cell surface expression is regulated by PDL1-AP, we performed a proteomic analysis of the cell surface in PDL1-AP knockout cells. Several proteins with a similar domain structure to PDL1 showed a decreased cell surface abundance. The functional consequence of PDL1-AP depletion was to relieve the suppression of T-cell anti-tumour immunity in human T-cell clones.

Conclusions
PDL1-AP plays a critical role in maintaining the stability of PDL1 at the cell surface of numerous cancer cell types and targeting PDL1-AP has beneficial effects similar to PD1 blockade in vitro. Effective targeting of PDL1-AP therefore offers a potential novel immunotherapy which complements current checkpoint inhibitor treatments.

Keywords
Programmed Death Ligand 1 (PD-L1)
Immunotherapy
Genetic Screen
Plasma Membrane

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Identification and localization of protein-pentanal adducts, a potential lipoxidation marker.

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Background: Oxidative stress has been linked to several inflammatory diseases; this may be due to oxidative damage to biomolecules. Oxidized phospholipids have been shown to be related to several pathophysiological pathways, as antigens or ligands, and more recently through protein modification creating protein-phospholipid adducts. The literature on protein-phospholipid adducts is sparse, although this type of protein modification could be of interest as potential biomarkers for disease. The aim of this project is to study the formation of these adducts and to uncover possible biomarkers, using both intact protein and bottom-up mass spectrometry techniques.
Methods: For this work, two model proteins were modified with pentanal, a saturated breakdown product of phospholipid oxidation and a model for phospholipid aldehydes, and the adducts were stabilized by reduction and analysed by ESI MS. The modified proteins were also subjected to SDS-PAGE, in-gel tryptic digestion, and LC-MSMS analysis.
Results: The top-down and bottom-up analysis showed the presence of pentanal adducts, and identified the sequence of 7 different modified peptides between the 2 model proteins. The mass shifts observed was consistent with the formation of Schiff's base adducts with pentanal, and only modifications to lysine were observed. Product ions resulting from the modified lysine immonium ions were identified.
Conclusions: Mass Spectrometry shows great potential as a tool for identification of diagnostic ions that can be used as biomarkers for inflammatory diseases. Future work will investigate adduct formation using oxidized phospholipids.
Keywords: oxidative stress, saturated aldehydes, lipid oxidation, lipoxidation

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Discovery, verification and pre-validation of novel candidate urinary biomarkers of prostate cancer

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Background
Prostate cancer (PCa) is a leading cause of death in men globally and has the highest incidence, mortality and 5 year prevalence rate amongst cancers in men of African descent. Even without therapy, PCa often runs a protracted natural history and many men die with rather than from PCa. Various candidate biomarkers have emerged via ‘omics-based approaches for use in diagnosis and monitoring of PCa, but there has been limited validation of these yet.

Methods
We carried out label-free proteomics on a Q Exactive to quantify significant differences in the urinary proteomes of African males (N=45) diagnosed with PCa, benign hyperplasia (BPH) or other prostatic disorders (‘disease controls’; DC). We also carried out serological analyses using a novel cancer-testis antigen microarray to identify distinctive patterns of autoantibody expression in these groups. We correlated these datasets and carried out in silico verification against Human Protein Atlas immunohistochemistry data. We furthermore carried out parallel reaction monitoring (PRM)-based pre-validation of our candidate PCa biomarkers.

Results
We identified 1545 urinary protein groups (9991 non-redundant peptides) (FDR<0.01). Unsupervised cluster analysis of the urinary proteomes classified the patients into their clinical groups; statistical analysis identified 73 protein groups as candidate PCa biomarkers. Serological analyses identified a further 24 potential diagnostic autoantigen biomarkers as upregulated in PCa patients compared to BPH or DC (FDR<0.01); three of these (FGFR2, COL6A1 and CALM1) were also differentially expressed in the urinary proteomes. In silico verification and PRM pre-validation identified the top 12 candidate urinary biomarkers of PCa, which demonstrated ethnic trends.

Conclusions
Our multi-omic discovery approach identified novel candidate biomarkers of PCa. Our in silico verification and pre-validation pipeline then allowed us to focus on the most promising of these candidates for further clinical evaluation.

Keywords
Prostate cancer, biomarkers, proteomics, protein microarrays, autoantibodies, in silico verification, parallel reaction monitoring.

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Swedish national infrastructure for biological and medical mass spectrometry

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Swedish national infrastructure for biological and medical mass spectrometry
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Background
A national infrastructure for biological mass spectrometry and proteomics BioMS, was started in Sweden in 2015. The BioMS consortium provides the Swedish Life Sciences community with state-of-the-art MS instrument and expertise.
Methods
The following technologies are available: HDX-MS, Proteogenomics, Targeted Proteomics, Chemical Proteomics, Lipidomics, Glycomics and Glycoproteomics.
Results
For the Swedish Life Sciences community, the access to high resolution instruments and novel technologies has increased. More interaction between different research groups in Sweden is one result of the BioMS consortium. Training and educational activities is performed in collaboration with the Swedish Proteomics Society and the Swedish Mass Spectrometry Society.
Conclusions
A geographically distributed infrastructure within Sweden is under operation and already supports several research projects and scientists. The need of high resolution MS support and expertise is significant not only in Sweden and BioMS is already engaged in several scientific projects from other countries.
Keywords
Infrastructure, HDX-MS, Proteogenomics, Targeted Proteomics, Chemical Proteomics, Lipidomics, Glycomics and Glycoproteomics.

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A pulsed SILAC-TMT multiplexing approach reveals protein synthesis and degradation differences on proteoform level

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The coordination of protein synthesis and degradation for regulation of protein abundance is a fundamental aspect of cellular homeostasis. Although mass spectrometry-based technologies nowadays allow for the determination of native protein turnover on a proteome-wide scale, proteoform resolution is often still missing. We investigated the feasibility of a workflow combining pulsed SILAC with TMT-labeling for separate evaluation of protein degradation and synthesis rates on peptide level.
HeLa cells were pulse labeled in four replicates using Lys0/8 and Arg0/10 SILAC medium. Peptides derived from 10 pulse time points were modified using TMT10plex reagents and measured on an Orbitrap Fusion Lumos in MS3 mode. Following database search using MaxQuant and data normalization, synthesis and degradation rates were estimated by fitting exponential equations to TMT intensities.
Synthesis and/or degradation curves were obtained for >55,000 sequences assigned to >7,200 protein groups. After data processing and filtering, protein synthesis and degradation rate constants received from different replicates showed good reproducibility. Moreover, half-lives were comparable to those reported with the standard pulsed SILAC approach. At the same time, our method circumvented missing values in the pulse time series leading to higher proteome coverage and enabling turnover determination on peptide level. The latter led to the recovery of differences in turnover of protein splice variants and posttranslationally processed proteins revealed by significant rate constant differences of peptides belonging to the same protein group.
Our data suggest that the original pulsed SILAC concept can be expanded using TMT labeling of different pulse time points. Moreover, this multiplexing strategy should also be applicable to disturbed, non-steady-state systems meanwhile rendering the separate determination of changes in synthesis and degradation rates possible. As a consequence, the challenging study of proteome dynamics under different biological settings can be facilitated, thereby eventually improving our global understanding of cellular proteostasis in health and disease.

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New tools to improve serodiagnosis of Brucellosis

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Background- Despite Brucellosis is eradicated from most of developed Countries, it still causes a public health impact and economic losses in animal husbandry in Mediterranean area. Nowadays, brucellosis is remerging as new challenge with outbreaks of both human and animal disease. Design of adequate strategies for preventing animal brucellosis and consequently human brucellosis requires understanding of microorganism and also of different strains. From diagnostic point of view, occurrence of false positive reactions in the serological tests available currently reduces their specificity. A "perfect" antigen has not been developed yet. Furthermore, the mass vaccination policy using Rev.1 vaccine, in many endemic areas of the Mediterranean with the aim of controlling/reducing disease prevalence in small ruminants, interfere with serological diagnosis of the disease. Proteome of Brucella spp is lack of consistency and so it is important set up protocols and methods to obtained proteins that are useful to develop rapid diagnostic test with immunoproteomics.
Methods- We analyze three strains of Brucella melitensis coming from library of National Reference Laboratory of Brucellosis (IZSAM- Teramo ITALY) in three technical replicates for each strain used a combined methods of bead beating and solubilization. After 2D electrophoresis, image analysis was performed with Progenesis same spots and proteins were digested with trypsin and then analyzed with MALDI-TOF/MS (Bruker Daltonics).
Results- Combined methods of extraction physical plus chemical were able to resolve huge numbers of proteins that can be used to set up immune-proteomics analysis to improve and complete serodiagnosis of brucellosis.
Conclusions- Proteomics is a useful approach to develop new diagnostic tools aimed to improve the outcome of Brucellosis eradication programs and to implement molecular epidemiology for a better understanding of spread and diffusion of brucellosis.
Work supported by BrucMedNet- ARIMNet2 - Coordination of Agricultural Research in the Mediterranean grant agreement no. 618127.
Keywords Brucellosis, diagnosis, vaccine

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A proteomics approach to decipher pneumococcal adaptation during CSF infection

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Background
To maintain fitness pathogenic bacteria such as Streptococcus pneumoniae have to adapt their physiology and virulence potential to changing host niche conditions during invasive infections. The virulence factor repertoire of pneumococci has already been deciphered (analysed in detail), but information on the proteome adaptation during invasive infection is very limited. Therefore, we used a label-free mass spectrometry approach to profile proteome adaptation of pneumococci during infection in a murine model.
Methods
Here we have employed an in vivo proteomics-based approach to identify pneumococcal factors contributing to cerebrospinal fluid (CSF) infections. A comprehensive and qualitative mass spectrometry (MS) spectra library was generated by TPP and SpectraST enabling bacterial proteome in vivo analyses even in the presence of a significant excess of eukaryotic proteins.
Results
A spectral library comprising 7,597 unique peptides corresponding to 1,165 proteins was assembled by measuring pneumococcal samples from different in vitro cultivations. Subsequently, approximately 200,000 pneumococci were recovered from the CSF of mice with pneumococcal meningitis by dual filter extraction. Orbitrap Velos MS including spectra to spectra comparison by SpectraST identified 685 proteins in control sample used for infection and 249 proteins from pneumococci recovered from CSF. The regulator ComDE and peptide transporter AliB were exclusively detected post infection. Subsequent testing in the meningitis model revealed that strains lacking AliB, ComDE or both displayed an attenuated meningeal inflammation and disease course compared to that induced by the wild-type strain.
Conclusions
The in vivo proteomics approach is a powerful tool to characterize protein dynamics of pathogens during dissemination in the host and enables identification crucial players involved in virulence or fitness.

Keywords
Pneumococci, in vivo proteomics, spectral library, meningitis model, virulence

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Extracellular Signaling in Neuroblastoma through Exosomes for novel drug Targets discovery

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Background: Neuroblastoma is the most common extracranial solid tumour in childhood comprising 8%-10% of all childhood cancers. The prognosis of patients with disseminated neuroblastoma is grim, with a 5-year survival rate of approximately 30%. Amplification of MYCN is one of the best genetic marker of risk in neuroblastoma. Indeed, its expression is strongly correlated with high aggressiveness and treatment resistance. Little is known about MYCN expression and extracellular environment in neuroblastoma.

Methods: Extracellular vesicles (EVs) were separated from neuroblastoma cell lines using differential ultracentrifugation. Flow Cytometry and Dynamic Light Scattering were used to characterize their morphology before large-scale mass spectrometry-based proteomics characterization of the EVs protein cargo.

Results: In this work we used the TET-ON/TET-OFF system to inhibit the MYC-N expression in SH-EP neuroblastoma cell line. We performed EVs isolation and morphological characterization before MS-based proteomic analysis. Quantitative proteomic analysis of EVs isolated from MYCN+ and MYCN- cell lines allowed us to identify 890 proteins. MYC-N expression induced the regulation of 152 proteins involved in several molecular networks such as Protein synthesis, Glycolysis and Extracellular matrix-Interactions.

Functional assays provide us a better knowledge about the metabolic alterations. We measured the lactate production between the cells with the MYC-N+ and MYC-N- detecting higher production of lactate in MYC-N+ cells.

Conclusions: This work suggests a strong relationship between MYC-N activation and exosome protein cargo modulation in neuroblastoma and opens new perspective on investigating the extracellular modulation in neuroblastoma.

Keywords: Neuroblastoma, exosomes, proteomics

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Biomarker validation using quantitative LC-MS combined with automated processing of human CSF and blood samples.

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A critical part of biomarker development is the validation and qualification of identified biomarkers. We have developed and utilized the Bravo AssayMAP platform for the semi-automated processing of cerebral spinal fluid and blood samples for biomarker validation and qualification. We analyzed CSF and plasma samples from the Australian imaging, biomarker and lifestyle study of ageing (AIBL), a longitudinal cohort investigating Alzheimer's disease. We found that the Bravo AssayMAP platform delivered reproducible sample preparation and allowed for the processing of over 400 samples per day. Using this work flow in combination with heavy isotope labelled peptides and standard flow chromatography we could accurately determine the level of over 40 CSF and 50 plasma proteins that have been implicated as biomarkers for the diagnosis of Alzheimer's disease. The workflow did not involve any depletion methods but we were still able to achieve limits of quantitation in 10's of ng/mL. We will present the comparison of sample processing with different AssayMAP cartridge chemistry (e.g. C18 vs PRP). Overall, we find that the AssayMAP system provided reproducible results with minimal manual intervention.

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Cross-linking mass spectrometry database searches: perspectives and strategies for validation and error assessment

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Background
Target decoy competition models are used to determine the false discovery rate in proteomics approaches including cross-linking mass spectrometry (XL-MS). Over the last several years, advances have been made in how to deal with the search of cross-links from an enlarged search space (the number of possible cross-links from a given database scales with the square of the number of cross-linkable peptides). Additional prior work has considered various filters for the data to restrict the number of accepted incorrect cross-link spectral matches (XSMs) due to the very small proportion of correct XSMs among all spectral matches that are scored.

Methods
We discuss and implement sophisticated target and decoy database construction methods and corresponding search methods to more precisely and accurately estimate the false discovery rate (FDR) and to maximize the number of XSMs that can be accepted at a fixed false discovery rate threshold. Various filters were screened for their effect on the overall search and will be discussed from both an experimental and a theoretical viewpoint. Search methods developed herein were applied to increasingly complex samples to demonstrate the capacity to increase confident identifications.

Results
We demonstrate increased precision and accuracy of FDR estimates and an increase in the number of identified cross-links from a complex sample using the new methods.

Conclusions
Minor mis-approximations in standard proteomics workflows can be detrimental to a large effect in XL-MS analyses. Careful considerations of parameters and methods can avoid leading to potentially large errors that might otherwise result in poor XL-MS results.

Keywords
cross-linking mass spectrometry; target decoy competition; decoy database;

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Protein Biomarker discovery in Alzheimer's disease using SWATH MS analysis

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Background; Alzheimer's Disease (AD) is fast becoming one of the largest global public health challenges, with an estimated 1 million people set to be diagnosed by 2025. Due to the progressive nature of the disease the cost of treatment and social care is set to cost £26.3 billion annually. However, biomarker discovery, specifically for Alzheimer's disease, is proving elusive due to the mixed pathology patients present with, as AD is difficult to distinguish between other forms of dementia such as Parkinson's and dementia with Lewy bodies. Biomarker discovery for clinical applications of diagnosis, prognosis and response to treatment requires a combination of mass spectrometry based proteomics platforms and immunological assays for validation/verification.
Methods; 90 AD and age and sex matched controls were prepped using a standard operating procedure for protein digestion. These samples were investigated using SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra), a novel data-independent mass spectrometry technique that produces a permanent digital record (SWATH map) bespoke to the sample. The data were analysed using OpenSWATH; one of the gold-standard processing tools in SWATH analysis.
Results; The top 5% of overexpressed proteins associated with AD were selected for further analysis, with a total of 25 proteins identified. Of these, 9 have not been previously described in the literature in relation to AD such as Maltase-glucoamylase, Keratin (type 1), Alpha-enolase and Alpha-mannosidase 2.
Conclusions; Several of our identified proteins have been described as playing a role in AD and the majority of these proteins were identified exclusively in the AD samples but not in the control samples so could be indicative of disruption of the Blood Brain Barrier in disease. These results could also lead to the creation of a panel of potential biomarkers of both known and novel proteins.
Keywords; Alzheimer's disease, Biomarkers, Mass spectrometry

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Proteomic insights in extracellular microvesicles from CSF and tears of multiple sclerosis patients

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Background: Multiple Sclerosis is multifactorial neurological disease characterized by great heterogeneity in clinical presentation. Emerging evidences showed that distinct types of brain cells release high number of Extracellular Vesicles (EVs), functioning as shuttles for the delivery of cargo among different cells within an organism. These data mean that EVs carry receptors, bioactive lipids, proteins, and nucleic acids by which EVs may modify the phenotype and functions of target cells. Widely investigated as biomarkers in oncology little is known about EVs roles in the Multiple Sclerosis. We carried out a proteomics characterization of EVs isolated from CSF and tears of Multiple Sclerosis patients.

Methods: CSF and tears from Multiple Sclerosis patients and patients with other neurological diseases were analyzed by a polychromatic flow cytometry method. Exosomes and EVs were sorted on the basis of their positivity to specific markers, by using a FACSAria III cell sorter (BD) and characterized by Dynamic Light Scattering (DLS). Proteomics data of pure microvesicles were obtained by LC-MS/MS system (Bruker) and subjected to web-based Ingenuity Pathway Analysis system to rebuild their functional implications in the physiology and molecular homeostasis of Multiple Sclerosis.

Results: high number of exosomes and microvesicles were measured in Multiple Sclerosis both in CSF and tears. We described a subpopulation of neuronal and microglia EVs in tears and, by proteomics approach, we characterized protein patterns of the most significant EVs subpopulations in both CSF and tears of patients.

Conclusions: on the basis of our knowledge this is the first time that extracellular vesicles and exosomes are described and characterized in tears, showing a subpopulation originated from neurons and microglia cells.

Keywords: Extracellular Vesicles, Multiple Sclerosis, Tears, CSF

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Detection of novel types of protein carbonylations in human plasma.

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Background
Accumulation of carbonylated proteins in cells and tissues is a hallmark of oxidative stress and is associated with ageing and age related diseases. In order to fully understand the role of protein carbonylation in health and disease, accurate identification of carbonylated protein(s), and their detailed characterisation is required.
Methods
We have used biotin-hydrazide and mass spectrometry based approach for identification of carbonylated peptides and proteins. We have used metal ion-catalysed oxidation to induce oxidation in single protein sample (bovine serum albumin) and we have analysed proteins from human plasma collected from healthy individuals. An in depth analysis of the peptide sequence data allowed us to identify 14 different types of carbonylated amino acids.
Results
In native human plasma we have observed 133 carbonylated sites in 36 proteins. The approach identified 10 hitherto undetected types of carbonylated amino acids in proteins: aldehyde and ketone modifications of leucine, valine, alanine, isoleucine, glutamine, lysine and glutamic acid (+14 Da), an oxidised form of methionine - aspartate semialdehyde (-32 Da) - and decarboxylated glutamic acid and aspartic acid (-30 Da). The carbonyl compounds reported are consistent with the chemistry of peroxyl and alkoxyl radicals generated on proteins.
Conclusions
The consequence of the formation of these carbonylation products has yet to be understood. However, it is important to note that some of these carbonyls can introduce changes to protein charge, give rise to Schiff base cross-links, and can lead to modification of residues that define protein structure (e.g. ring opening of P residues). These events may affect protein function (e.g. interaction with other proteins) or protein conformation and activity.
Keywords
Protein oxidation, Biotin-hydrazide, Metal ion-catalysed oxidation, Oxidative stress

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Reactome Interactive Pathway Analysis

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Background
Reactome (http://reactome.org) is a free, open-source, curated and peer-reviewed knowledge base of biomolecular pathways. Pathways are arranged in a hierarchical structure that largely corresponds to the GO biological process hierarchy, allowing the user to navigate from high level concepts like immune system to detailed pathway diagrams showing biomolecular events like membrane transport events or phosphorylation.

Methods
We performed comprehensive user experience testing with external users not familiar with the Reactome web site, which identified shortcomings in website navigation and presentation of the complex Reactome content. In response to this analysis, we have refactored the Reactome web site, introducing new visualisation concepts and approaches. We have also invested significantly in performance optimisation, ensuring a fully interactive user experience even for the analysis of complex genome-wide datasets with thousands of identifiers.

Results
For navigation through the higher levels of the pathway hierarchy, Reactome now provides scalable, interactive textbook-style diagrams, which are also freely downloadable and editable in SVG format. Repeated diagram elements like ‘mitochondrion’ or ‘receptor’ or are also available as a library of currently more than 300 graphic elements. Detailed lower-level diagrams are now downloadable in editable PPTX format as sets of interconnected objects.

Conclusions
The Reactome pathways resource provides user-friendly, highly interactive analysis of complex ‘omics datasets. Well-documented software components are available as open source, standalone Java widgets. High quality, textbook-style illustrations facilitate navigation through the Reactome pathway space, and are also available for download as a community resource, including the Reactome Icon Library of more than 300 graphical elements for pathway visualisation.

Keywords
Pathway analysis, Visualisation, Networks, Java.

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Inactivation of endogenous tissue proteases by brief exposure to high hydrostatic pressure.

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Sample preparation for proteomic analysis of whole tissues requires extraction of the entire intact proteome followed by specific digestion with enzymes such as trypsin. The presence of endogenous tissue proteases can lead to non-specific proteolysis during sample preparation. To prevent this, many protocols call for the addition of protease inhibitors. While inhibitors can be quite effective, their presence and continued action can result in poor trypsin digestion and fewer peptides available for analysis. Earlier studies have shown that very high pressure can be used to inactivate sample-derived enzymes. In this study, we examined the utility of high hydrostatic pressure for rapid inactivation of endogenous proteases in tissue lysates.
Various tissue lysates were exposed to high hydrostatic pressure, up to 90,000psi (6.2kBar), using a HUB880 Explorer high pressure instrument (Pressure BioSciences, Inc). Endogenous protease activity was assayed by total protein separation on SDS-PAGE gels after overnight incubation of treated and control samples at 37°C. Compared to controls, the pressure-treated samples showed a significant decrease in overall proteolysis suggesting that endogenous proteases had been irreversibly denatured and inactivated. The effects of different pressure levels (50,000-90,000psi) and time at pressure (2-40mins) were also examined. Our preliminary data strongly suggest that the activity of endogenous proteases can be significantly reduced or eliminated by high pressure treatment, without the need for protease inhibitor cocktails. SDS-PAGE analysis clearly shows that whole tissue lysates treated at 90,000psi for as little as 2 minutes, exhibit significantly less protein loss compared to samples kept at ambient pressure.
Brief, high pressure pre-treatment may enable better proteomic analysis of complex tissue samples, by reducing the presence of semi-tryptic and non-specific peptides generated by residual activity of endogenous proteases. Further studies are being conducted to optimize pre-treatment parameters for maximal coverage and quantitative analysis of tissue samples in proteomic studies.

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microDIA acquisition and software enables identification of peptides in pseudo-SRM MS/MS spectra from sequence databases

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Background

Typical data independent acquisition (DIA) approaches use wide isolation windows that produce noisy MS/MS data and require sample specific spectral libraries to be generated for confident peptide identification. Large DIA isolation windows have the drawback of complicating peptide modification analyses – especially when modified and unmodified versions of the peptides are co-isolated. To address these limitations, we developed the microDIA workflow that uses sequentially overlapping precursor isolation windows combined with an automated MS/MS spectrum deconvolution method. It produces highly specific MS/MS spectra with ± 1 m/z mass-isolation windows across the precursor range spanning most detectable tryptic peptides in a single shot analysis. The workflow is implemented in the ProtalizerTM software platform, that enables direct peptide and protein identification without requiring a spectral library.

Methods

We acquired data using 6 m/z wide DIA windows, each overlapping 2 m/z and covering a 430-910 m/z mass range with a 37 Hz scan rate using an Impact II UHR-Q-TOF coupled to a nano Elute UHPLC (Bruker Daltonics). Hela tryptic digest or mixtures of Hela, Yeast and E.Coli lysates triptic digests -according to Navarro et al (2016) were separated with a 2h gradient. ProtalizerTM software (Vulcan Analytical) was used to identify peptides and proteins from a hybrid Swiss-Prot reference database of Yeast, E. coli, and Human proteomes. Quantification was performed via MS2 chromatograms.

Results

We increased the number of protein ID’s by 64% compared to a standard shotgun workflow at 1% Protein FDR. Median CV values observed on replicates of the three proteome mixtures were below 10%. Details illustrating the quantitation and comparative dynamic range accessible with shotgun and microDIA approaches will be given.

Conclusion

Our results illustrate the ability to perform in-depth DIA protein identification and quantification without creating a spectral database beforehand.

Keywords:

- Sequence database
- Data-Independent Acquisition
- Identification

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A Lysis-free, Vesiculation-based Methodology to Isolate High Purity Cell Membranes for Proteomics and Lipidomics Applications

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Background
Global profiling of membrane proteins and lipids by mass spectrometry aids in qualitative and quantitative characterization of plasma membrane composition. Isolation of pure membrane fraction has always been a challenge and conventionally it has been achieved by subcellular fractionation of cell homogenate. We have employed a vesiculation-based method for isolation of pure membrane fraction from cultured cells, where there was no requirement to lyse the cells but yields high purity membrane fractions in an easy and hassle-free approach.
Methods
The cells were chemically induced to vesiculate by perturbing the cytosolic anchoring of membranes resulting in budding off cell membrane as giant plasma membrane vesicles (GPMVs) into the media. GPMVs are harvested from the suspension while cells remain adhered on the dishes. The purity of membrane preparations was assessed by immunohybridization against membrane or organelle specific markers and were compared with conventional ultracentrifugation aided membrane preparations. The proteome and lipidome profile of GPMVs were analyzed by LC-ESI-MS/MS.
Results
GPMVs were of 1-2 µ in size and showed an enrichment of membrane specific marker Na/K ATPase similar to conventional membrane isolates. However organelle specific markers for ER, golgi and mitochondria were clearly depleted in GPMVs indicating the purity of membrane preparations. The protein and lipid profiles of GPMVs and conventional membrane isolates were comparable. Enrichment of proteins associated with membranes, particularly those in focal adhesion, cell-cell adherence and extracellular vesicles were evident in GPMV preparations. GPMVs also showed an enrichment of membrane phospholipids and a depletion of storage glycerolipids as expected. The method was further validated by employing to capture the differences in membrane lipid composition of Phosphatidylserine auxotrophs.
Conclusion
Membrane isolation from cultured adherent cells by vesiculation proves to be an easy alternative methodology to yield pure membrane preparations without intracellular contaminations for high throughput omics applications.

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Large Scale Quantitative Urine Proteomics of Japanese Healthy Volunteers

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Background
Urine is an important biological fluid and accessible noninvasively for biomarker discovery and health checkup. We started "All-in-One" Urine Test project to make above aims only by urine examination. A large-scale urine proteomics are conducted to discover biomarkers for early disease detection or monitoring health conditions. Since urine proteomics may provide standard references among individuals, genders, ages as profiles of healthy volunteer.

Methods
180 urine samples from both male and female Japanese health volunteers of age from 10's to 60's were collected and stored at -20°C until use. Optimized Methanol/Chloroform precipitation method used to extract urine proteins. 500ng of purified tryptic urine peptides of each specimen was analyzed twice in SCIEX 5600+ MS. IDA and DIA (SWATH-MS) were performed. Consensus library was created from IDA results searched by ProteinPilot™. SWATH data analysis was done by Micro application of SWATH in PeakView™ as normal processing settings. XIC extract window is 10 min. with 50ppm width of precursor m/z. Processed peaks intensity of peptide and protein were reported.

Results
A Crude urine protein concentration was variable in all Japanese health volunteers. In total, 1880 urine proteins were identified with 1% global FDR with over 34000 distinct peptides. 212 (11.3%) unique proteins were identified from children's urine. There is minimized diff (16 proteins) in 40's between female and male. Maximum diff was observed in 60's urine samples. 308 proteins changed significantly ($p<0.01$). 29 proteins changed significantly ($p <0.001$) in all urines, Leukocyte elastase inhibitor show high concentration in female urine.

Conclusions
Quantitative analysis results of urine proteins and peptides of Japanese health volunteers were summarized as a standard reference in our center to identify urine biomarkers for diseases or health disturbance in the future. We hope the results can provide an indispensable background of urine biomarker discovery research.

Keywords
Urine Proteomics, SWATH-MS.

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Cell type-specific proteogenomics in the infarcted heart

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Background

Myocardial infarction (MI) is one of the cardiovascular diseases with leading cause of death worldwide. Multiple molecular and cellular mechanisms are involved in cardiac remodeling after ischemia-reperfusion (I/R) injury. The present study aimed at determining the cell type-specific functional contribution to post-MI heart failure development, intercellular crosstalk and the cells-serum communication in a pig model of I/R.

Methods

Acute MI was induced in large white swine using a closed-chest 30 min I/R, percutaneous technique (day 3 and day 7 post-I/R). Pigs sacrificed after baseline imaging served as controls. Cardiomyocytes (CMs), fibroblasts (FBs), macrophages (MOs) and endothelial cells (ECs) were isolated from cardiac tissue after sacrifice, the last three types via flow cytometry sorting, for further mRNA and protein expression analysis. Blood samples were collected and processed for proteomics.

Results

A high-throughput mass spectrometry-based proteomics and RNASeq-based transcriptional characterization demonstrated that cardiac cells not only were mutually involved in a set of common processes (apoptosis, protein biosynthesis, response to oxidative stress, mitochondrial energy metabolism, among others), but also showed cell type-specific functions. Within the first week after cardiac insult, surviving CMs recovered mitochondrial and contractile proteins; FBs' molecular phenotype switched into extracellular matrix-degradating myofibroblasts; MOs displayed pro-inflammatory M1-like to anti-inflammatory M2-like gene signatures conversion and ECs' mRNA profiles were mostly implicated in angiogenetic processes. Altogether, transcriptomics and proteomics data provided a comprehensive resource of intercellular crosstalk and revealed a panel of proteins transmitted from different cardiac cells to blood serum over the time course post-I/R.

Conclusions

Our data constitute a comprehensive repository for the identification of cell type-specific molecular factors that contribute to post-I/R injury. Future cardioprotective therapies should benefit from the improved understanding of the molecular events underlying cardiac repair that can be derived from this knowledge.

Keywords

myocardial infarction; heart cells crosstalk; serum; proteomics; transcriptomics

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Integrative omics profiling within the Swedish SCAPIS SciLifeLab (S3) Wellness Profiling program

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Background
The Swedish SCAPIS SciLifeLab (S3) Wellness Profiling program is based on the Swedish CArdioPulmonary bioImage Study (SCAPIS), a large prospective clinical study involving 30,000 individuals with extensive clinical phenotyping, as well as on the Human Protein Atlas project (www.proteinatlas.org), where a combination of genomics, transcriptomics, proteomics and antibody-based profiling is used to study the global protein expression patterns in human cells, tissues and organs.

Methods
In the ongoing pilot study, 100 participants are followed longitudinally every three months after their baseline examination with repeated analyses of molecular markers in blood, urine and stool samples in combination with physical measurements and continuous monitoring of biological signals like sleep and activity. The samples are analyzed in a large number of platforms including several complementary proteomics methods (Olink PEA, antibody bead arrays, autoimmunity profiling and targeted proteomics), immunology (CyTOF), genomics (WGS), transcriptomics (RNA sequencing), microbiome analyses (16s RNA and metagenomics) and metabolomics (plasma/urine/lipids). In addition, the data collection also consists of extensive lifestyle and imaging data from SCAPIS including MRI, CT-scans and ultrasound analysis.

Results
We have studied the longitudinal effects of lifestyle variation in a healthy population based on personalized expression profiles from different platforms including transcriptomics and proteomics. Using multivariate methods, we are also integrating clinical diagnostics, activity and immunology data with omics data to model early signs of atherosclerosis and other diseases.

Conclusions
A large collection of data from multiple platforms is used for integrative studies to understand the normal variation of molecular profiles in healthy individuals over time with the goal to facilitate a molecular definition of health.

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Serologic profiling of the Candida albicans cell surface-associated proteome upon dimorphic transition in invasive candidiasis

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Background. The ability of many Candida species to reversibly switch between yeast and hyphal growth under specific host environmental stimuli (dimorphism or dimorphic transition) is important for virulence of Candida albicans. Both morphological forms are pathogenic, and may promote different stages of the infectious process and expose distinct host recognition biomolecules at their surfaces. A better knowledge of their cell surface antigens could offer a rationale for the future design of new diagnostic and therapeutic strategies for invasive candidiasis (IC), an opportunistic and life-threatening mycosis.

Methods. We examined IgG antibody responses to the C. albicans cell surface-associated proteome upon dimorphic transition in IC patients using serologic proteome analysis (SERPA) and data mining tools.

Results. A total of 27 cell surface-associated proteins (CSPs) were differentially immunodetected in yeasts and hyphae during IC. Capture ELISAs on selected CSPs confirmed SERPA data. Two-way hierarchical clustering analysis unveiled two IgG antibody-reactivity signatures that segregated IC sera hybridized with yeast and hyphal CSPs into two discrete groups. Coordinated IgG antibody responses to two repertoires of CSPs as a function of the growth form were evidenced in IC. Pairwise correlation and gene ontology analyses revealed distinct subsets of functionally related CSPs that showed opposing IgG antibody-mediated immune recognition patterns in yeasts and hyphae during IC. Changes in the antigenicity of the 27 identified CSPs upon dimorphic transition in IC induced topological differences in their immune co-recognition networks. By testing the effect of CSP abundance normalization on reactivity changes, we found that antigenicity of CSPs was modulated in IC by changes in their relative abundance and potential post-translational modifications in their epitopes upon dimorphic transition.

Conclusions. These findings provide new insight into the host immune recognition of the C. albicans yeast and hyphal surface-associated proteome in IC.

Keywords. Cell surface, antigens, immunoproteomics, yeast, hypha

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The Draft Human Proteome as of 2017: Metrics from the HUPO Human Proteome Project

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Background: The HUPO Human Proteome Project (HPP) continues major progress toward its two goals: (1) completing the protein parts list, with the annually-updated draft human proteome, and (2) making proteomics an integrated complement to genomics and transcriptomics throughout life sciences research.

Methods: The HUPO Human Proteome Project is a global collaboration of 50 teams organized by chromosome, disease category, and resource pillars for protein capture, mass spectrometry, and bioinformatics. Investigators worldwide contribute primary data and metadata through ProteomeXchange to PRIDE, PASSEL, MassIVE, and jPOST. PeptideAtlas and GPMDB reanalyze all the data with standardized pipelines. neXtProt curates all protein evidence. Human Protein Atlas provides extensive data on tissue and intracellular expression of proteins and transcripts. The baseline data for 2017 are neXtProt 2017-01-23 and PeptideAtlas 2017-01.

Results: neXtProt 2017-01-23 has 17,008 confident protein identifications (Protein Existence [PE] level 1), up from 13,664 in 2012-12 and 16,491 in 2014-10, compliant with the HPP MS Guidelines v2.1 (https://hupo.org/Guidelines). There were 2579 “missing proteins” (PE2+3+4) as the baseline this year. PeptideAtlas 2017-01 (updated 2017-04-20) has 15,173 canonical proteins. GPMDB has 16,190 EC4 entries. Each has extensive data on PTMs, single amino acid variants, and splice isoforms. Human Protein Atlas displays spatial evidence of tissue and subcellular expression of proteins and transcripts. The B/D-HPP has generated organ-specific popular protein lists for quantitative targeted proteomics studies.

Conclusions: 87% of the predicted PE1-4 protein-coding genes in neXtProt now have high confidence protein-level evidence. The C-HPP has accelerated efforts to identify missing proteins, assisted by missingproteinpedia.org and proteogenomic analyses. Identification and characterization of sequence variants, splice isoforms, and PTMs represent major opportunities to link structure and functions of proteins.

Keywords: HUPO Human Proteome Project, neXtProt, PeptideAtlas, Human Protein Atlas, missing proteins

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Proteome Analysis of Alveolar Epithelial Type II Cells in Response to Pulmonary Aspergillus fumigatus Infection

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Aspergillus fumigatus is a ubiquitous mold and a severe threat for immunosuppressed patients as it can induce highly lethal invasive aspergillosis. A. fumigatus conidia are airborne and once inhaled, reach the alveoli, where they encounter alveolar epithelial cells (AEC). Majority of the previous studies reported the importance of the surfactant-producing AEC II during A. fumigatus infection via in vitro experiments using cell lines. For this study we established a negative magnetic isolation protocol yielding untouched primary murine AEC II with a purity >90 %, allowing ex vivo analyses of the cells, which encountered the mold in vivo. Subsequently, we performed label-free proteome analysis of AEC II isolated from mice 24h after A. fumigatus or mock infection. We quantified 2256 expressed proteins and found 154 proteins to be significantly differentially abundant between both groups (ANOVA p-value ≤ 0.01, Fold Change ≥ 1.5, quantified with minimum 2 peptides). The majority of these proteins were higher abundant in the infected condition, thereby reflecting a comprehensive activation of AEC II upon interaction with A. fumigatus conidia. This activation was especially represented by an enrichment of the proteins related to oxidative phosphorylation and consequently energy production. However, the most strongly induced protein was the L-amino acid oxidase (LAAO) Interleukin 4 induced 1 (IL4I1) with a 42.9 fold upregulation (ANOVA p-value 2.91- 10). This enzyme has previously been found in B cells, macrophages, dendritic cells and rare neuronal cells. IL4I1 upregulation in AECII was confirmed by qPCR, Western blot and immunohistology. Since LAAO are key enzymes for the production of bactericidal products, AECII might actively participate in pathogen defense. This study provides insights into proteome changes of primary AECII thereby opening a new avenue to analyze the molecular changes of this central lung cell upon infectious threats.

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Phosphoproteome profiling of different clinical phases in multiple myeloma

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Background: Multiple myeloma (MM) is the second most common blood cancer. Although the life expectancy of patients diagnosed with MM has gotten better during the past 15 years due to new therapies, the majority of patients eventually relapse and become resistant to existing therapies, ultimately leading to their death. Thus, there is a demand for more potent therapies to treat MM patients, and for better methods to monitor MM progression. To meet these demands a deeper understanding of MM disease progression mechanisms is needed. The aim of this work is to elucidate the changes in protein phosphorylation in the different clinical phases of MM. Known signalling pathways deregulated in MM include NF-kB, MAPK, PI3K/Akt/mTOR, and Jak2/Stat3 pathways. They are especially important for MM because of their roles in cell proliferation, survival, resistance to apoptosis, and drug resistance.

Methods: Quantitative LC-MS/MS-based phosphoproteomics on malignant plasma cells (CD138+) from MM patients is done in combination with bioinformatic analysis. CD138+ cells are enriched from MM patient bone marrow aspirates. Phosphorylation sites are confirmed using PhosphoRS and quantitative phosphoproteomics analysis will be done using label-free quantitation.

Results: The project is at an early phase. Optimization of phosphopeptide enrichment from MM patient samples has only just recently been completed. The focus in the analysis of the phosphoproteomic data will be at first in comparing the possible differences in the NF-kB, MAPK, PI3K/Akt/mTOR, and Jak2/Stat3 pathways between the different clinical phases of MM. Of interest will also be the HDAC, WNT, HSP, NOTCH and Hedgehog signalling pathways, which have also been implicated in having a role in MM.

Conclusions: This project will hopefully assist in the development of more potent therapies to treat MM, and also in the identification of novel biomarkers for MM to assist in better monitoring MM progression.

Keywords: phosphoproteomics, multiple myeloma

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Immunomodulatory Effects of Peritoneal Alanyl-Glutamine in Clinical Peritoneal Dialysis Detected by a Multi-Omics Biomarker Approach

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Background: Peritoneal dialysis (PD) is a life-saving renal replacement therapy for patients with end-stage renal disease. PD-effluent represents a rich source of biomarkers for predicting outcome and monitoring therapy. Novel PD-fluids may provide patient-tailored benefits, such as peritoneal immunomodulation. Although biomarker information could improve the management of PD patients, high abundance plasma proteins currently hamper the potential of PD-effluent as a source of biomarkers.

Methods: A multi-omics approach was established utilizing biomaterial obtained from two randomized clinical trials (RCT), based on enrichment of low abundance proteins and highly sensitive identification and quantitation using tandem mass tags (TMT) or RNAseq based transcriptome analysis of effluent cells. PD patients were treated either with standard PD fluid or with added alanyl-glutamine (AG). PD-effluent samples were depleted from high abundance plasma proteins using a bead-coupled combinatorial hexapeptide-library. Enriched low abundance proteins were then subjected to TMT-labeling and filter-aided sample preparation liquid chromatography mass spectrometry (FASP-LC-MS).

Results: The presented workflow identified more than 2500 unique proteins in contrast to only 140 unique proteins detected in PD-effluents in literature, resulting in a significantly increased coverage of the PD-effluent proteome. Identified proteins provided novel information on overrepresentation of proteins linked to membrane remodeling and fibrosis, whereas underrepresented proteins indicate decreased immuno-competence. Addition of AG resulted in changes of markers, reflecting peritoneal immune-modulation in accordance with transcriptome analysis. Integration of transcript data from almost 10000 genes indicated reduction of fibrosis and increased host defense as candidate mechanisms.

Conclusions: Our data suggests feasibility of multi-omics approaches to investigate cell derived biomarkers for pathomechanisms relevant in PD. Treatment with AG was associated with restoration of biological processes involved in important immune processes and reduction of fibrosis. These biomarkers may serve as surrogate for evaluation of novel interventions in PD and for monitoring PD therapy.

Keywords: Renal replacement therapy, plasma proteomics

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Functional analysis in pulmonary Systemic sclerosis

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Systemic sclerosis (SSc) is a rare and severe connective tissue disorder. In particular, pulmonary involvement of SSc mainly comprises characteristics of interstitial lung diseases (SSc-ILD) and presents pulmonary arterial hypertension (PAH). Although, much is known on onset, development and damage of Systemic sclerosis, to date a complete successful pulmonary therapy is not yet available. Interestingly, improvements in sampling of molecules that coat normal and diseased airways, led to bronchoalveolar lavage (BAL) selective of the respiratory tract for comparing changes, normal/pathologic. For this reason, in order to ameliorate the knowledge about changing in SSc lung environment, we performed a differential proteomic analysis of BAL samples from SSc patients, smoker and no-smoker controls. The differential expression pattern elaborated by Principal Component Analysis highlighted the specific SSc protein profile with respect to controls and the enrichment analysis shed light on process networks where the identified proteins are involved such as inflammation induced by IL6 signaling, complement system, Jak-STAT, kallikrein-kinin system and innate inflammatory in addition to blood coagulation, immune response by phagocytosis and phagosome in antigen presentation. In particular, MetaCore network suggests 1433epsilon and S10A6 as upstream molecules regulating the highlighted processes. Numerous findings report their involvement in fibrosis, innate immunity and vascular damages, suggesting their role as potential pharmacological targets.

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Impact of peritoneal dialysis fluid on O-GlcNAcylation of the mesothelial cell proteome and cell survival

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Background: The renal replacement therapy with peritoneal dialysis (PD), an alternative to hemodialysis, removes toxins, water and solutes from the uremic patient by using PD-fluids (PDF). Due to their physicochemical properties, including high glucose content, glucose degradation products, unphysiological buffer and low pH, PDF harm the mesothelial cells of the peritoneum, which are used as dialysis membrane. The hexosamine biosynthetic pathway (HBP), which mediates the post-translational modification of proteins with O-GlcNAc, is a specific glucose- and glutamine-dependent cell mechanism and was recently described to mediate cytoprotection and cell survival in PDF-treated mesothelial cells. The aim was to identify PDF-induced differences of O-GlcNAcylation on the single protein level.

Methods: Human mesothelial cells were incubated with HBP modulators and PDF. A 2D-gel and 2D-Western blot based screen was employed to detect treatment induced differences of proteins modified with O-GlcNAc. Protein identification following O-GlcNAc specific immunoprecipitation and trypsin in-gel digestion was performed using a MALDI LTQ Orbitrap. The impact of the modulation of O-GlcNAcylation on the outcome after PDF treatment was also monitored using live/dead fluorescence staining.

Results: In total, 493 protein spots could be detected, of which 22% were found to be O-GlcNAcylated. Exposure of mesothelial cells to PDF leads to significant increase of O-GlcNAcylation. Inhibition of O-GlcNAcylation is associated with decreased viability. Increased levels of O-GlcNAc lead to improved viability of cells after PDF exposure. Whereas the total proteome shows limited changes in protein abundance, O-GlcNAc-specific 2D-Western blots reveal significant differences of specifically O-GlcNAcylated proteins after treatment with PDF.

Conclusions: The relevance of O-GlcNAc-specific changes of the proteome for mesothelial cell survival demonstrates the therapeutic potential of modulation of O-GlcNAcylation to increase endogenous cell protection during PD. Therefore these findings may lead to better understanding of negative molecular effects on mesothelial cells during PD and improvement of the therapy.

Keywords: O-GlcNAcylation

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Simple, scalable and ultra-sensitive tip-based identification of protease substrates

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Background
Large-scale N-terminomics has revolutionized protease research, but is disseminated only in few labs. Our novel ChaFRAtip approach requires only basic protein chemistry, a pipette tip with a cellulose frit, SCX particles and simple buffers. We applied ChaFRAtip to study dynamic proteolytic cleavage during apoptosis with unprecedented sensitivity.

Methods
We optimized tip-based fractionation of labeled/unlabeled tryptic cell lysates based on theoretical peptide net charge states at pH 2.7.
Using staurosporine-treated SH-SY5Y cells we compared ChaFRAtip with our previous HPLC-based ChaFRADIC approach.
Using ChaFRAtip we studied the dynamics of proteolysis during staurosporine-induced apoptosis at different time points (0/1.5/3/6 h). Samples were labelled with iTRAQ-8plex, pooled, digested with trypsin and ChaFRAtip was conducted in 6 technical replicates by two different individuals.
Samples were analyzed by nano-LC-MS/MS. Data were analyzed using Mascot, Proteome Discoverer and Percolator at 1% FDR.

Results
We obtained fractions highly enriched (>85%) in peptides of specific net charge states (+1,+2,+3,+4) in a reproducible and scalable manner (10/50/200 µg starting material).
Applying the complete ChaFRAtip workflow we achieved a similar reproducibility and sensitivity as with the HPLC-based approach, at a fraction of the costs.
Studying the dynamics of apoptosis, we quantified 2000±57 unique N-terminal peptides representing only unique protein entries with a strong correlation across biological and technical replicates – notably, from only 6 µg per sample (amino acid analysis quantified), corresponding to 357 quantified N-termini per µg.
Using stringent cutoffs, after 1.5 h of staurosporine treatment 81 protease substrates were identified, followed by 109 and 30 additional substrates after 3 h and 6 h, respectively. These substrates clearly confirm the expected caspase activity and furthermore an orchestrated regulation of specific pathways.

Conclusion
ChaFRAtip allows the highly sensitive identification of proteolytic targets and consensus motifs. It is straightforward, low cost, and requires only minimal standard equipment.

Keywords: Proteolysis, Apoptosis, N-terminomics

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Identification of potential biomarkers for vivax malaria using clinical isolates: A Proteomics intervention

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Background
Plasmodium vivax contributes to 41% of the global malaria burden outside Africa and is the most widespread human malaria parasite. Its detection primarily depends on light microscopy and specific rapid diagnostic tests. Although inexpensive and robust, these tools are incapable of detecting malaria at low parasitemia, a characteristic feature of vivax malaria. Furthermore, these approaches cannot accurately diagnose mixed infections and warrants the need for sensitive diagnostic tests for prompt malaria surveillance.

Methods
A comprehensive proteomic analysis of 10 P. vivax clinical isolates was performed to identify highly abundant parasite proteins for evaluation as serodiagnostic antigens. Parasites were isolated using saponin lysis and the proteins extracted were processed and subjected to LC-MS/MS analysis using Q-TOF mass analyzer.

Results
An overall proteome coverage of 297 unique P. vivax proteins was obtained. While a major proportion of the P. vivax proteins were either hypothetical or involved in basic cellular activities, few proteins such as such as tryptophan-rich antigen (Pv-fam-a; PVX_090265), Pv-fam-d protein (PVX_101520), Plasmodium exported protein (PVX_003545, PVX_003555) and hypothetical protein (PVX_083555) were detected in more than 60% of the ten clinical isolates and found to be unique to P. vivax without orthologs in P. falciparum. These proteins represent a unique list of P. vivax proteins that may be explored further as antigens for the serodiagnosis of vivax malaria.

Conclusions
In this study we report five P. vivax parasite proteins as probable biomarkers for vivax malaria. Their absence in P. falciparum makes them ideal candidates for malaria diagnosis. Although the efficacy of their serological effectiveness is yet to be tested, our findings provide new avenues to tackle the challenges of vivax malaria diagnosis

Keywords
Mass spectrometry, P. vivax proteome, biomarkers, serodiagnosis

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Characterization of NLRP3 inflammasome signaling pathway by proteomics

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Background
Inflammasomes are multimeric protein complexes formed during innate immune response. The pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The inflammasomes are related to various inflammatory diseases. However, whether inflammasome signaling affects the secretion of exosomes and the detail mechanisms of activation of NLRP3 inflammasome signaling mains to be determined.
Methods
We isolated the exosomes from macrophages with treatment of mock, endotoxin or endotoxin/ nigericin. A label-free quantification method by MS/MS was used to identify the components of exosomes. The dynamic change of protein complexes of NLRP3 were investigated by IP-MS.
Results
Totally 2331 proteins were identified and 513 proteins were exclusively expressed in exosomes with endotoxin and nigericin treatment. The differentially expressed proteins were classified by Gene Ontology and KEGG pathway. The immune response related proteins and signaling pathways were specifically enriched in inflammasome-derived exosomes. Moreover, we treated macrophages with the exosomes from different stimulation. The potential mechanisms of novel interactors of NLRP3 was further verified in BMDMs.
Conclusions
We found that inflammasome-derived exosomes directly activate NF-κB signaling pathway, while the control or endotoxin-derived exosomes not. The inflammatory signaling was amplified in neighbor cells in an exosome-dependent way. The inflammsome-derived exosomes might be used to augment the immune response in diseases treatment and prevent of the transfer of these exosomes might cure auto-immune diseases.
Keywords: exosomes, inflammasome, NLRP3, protein complex

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HDMSE proteomic analysis of the mode of action of short chain fatty acids in colon

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Short-chain fatty acids (SCFAs - including butyrate and propionate) are bacterial metabolites produced during fermentation of dietary fibre and probiotics in the colon. SCFAs affect cellular processes at proteomic and transcriptional levels, due to regulation of protein lysine acetylation. The differential effects of butyrate and propionate were evaluated at the level of metabolic control by proteomic evaluation of a mitochondrial-enriched fraction, using a HDMSE label free workflow. 1453 proteins were identified (two biological and technical replicates/sample), of which 1380 were identified and relatively quantified by 3 or more peptides. Our results show that whilst these SCFAs have some functional overlap, there are distinct, differential effects, including synergistic and inhibitory interactions on specific metabolic enzymes. Analysis of lysine acetylation indicated acetylation of enzymes associated with energy metabolism: glycolysis and the tricarboxylic acid cycle. The altered levels and potential activities of metabolic enzymes has informed our approach to modelling metabolic control by SCFA. These data support distinct physiological roles for SCFAs in colon epithelial function, suggesting that SCFAs act not only as single components, but also have distinct effects acting in combination. Balancing their relative levels may be critical in design of cancer therapeutics.

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Proteome remodelling in senescent peritubular cells of human testis

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Background
Male reproductive functions decline with increasing age. A consequential reduction of spermatogenesis in older males may be attributed to a deteriorated capacity of the spermatogonial stem cell (SSC) niche in its function to provide a suitable microenvironment for SSC activity. It has emerged that human testicular peritubular cells (HTPCs), which build the wall of seminiferous tubules, constitute an important component of the SSC niche. However, the extent of cellular senescence of peritubular cells and potential consequences for ageing of the SSC niche is so far unknown.
Methods
Using nano-LC-MS/MS based label-free quantitative proteomics, we analysed HTPCs and the corresponding secretome anticipating it may represent the senescence-associated secretory phenotype (SASP) of in vitro-aged cells (i.e. from advanced passages) and pre-senescent cells (early passages) obtained from seven individual men.
Results
We profiled more than 3,000 proteins and found comprehensive age-associated abundance changes in the cellular proteome and the corresponding secretome of HTPCs. Beside known hallmarks of ageing, highlighting the validity of our approach, proteins so far not mentioned in this context showed extensive abundance alterations in senescent HTPCs. While proteins involved in cell redox homeostasis and response to reactive oxygen species increase, mitochondrial matrix proteins and proteins participating in extracellular matrix organisation are reduced in senescent HTPCs. Age-associated changes in the secretome suggest a realignment of the paracrine signalling network and could particularly influence the communication between peritubular cells and adjoining SSCs. It is therefore conceivable that quantitative alterations of secreted factors can lead to an age-related reduction of SSC niche function. This proteome imbalance emphasises the relevance of the SASP of peritubular cells for surrounding cell populations and maintenance of homeostasis in human testis.
Conclusions
This study contributes to the understanding of mechanisms of testicular ageing and its consequences for male reproduction.
Keywords
Aging, testis, peritubular cells, stem cell niche

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All That's Listed Does Not Code

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Background
The annotation of the human genome is in a permanent state of flux. While the protein coding complement has been relatively stable in absolute numbers over the last few years, genes classified as coding in the three main reference proteomes (Ensembl/GENCODE, RefSeq and UniProtKB) are reclassified with each new release.

Previously we analysed the manually annotated GENCODE human reference set using peptide evidence from seven large-scale MS analyses. We defined a set of 2,001 coding genes with very little proteomics support and features that suggested they were not protein coding [Ezkurdia et al, HMG, 2014]. Manual annotators have since either withdrawn or reclassified 908 genes that were previously classified as coding.

Methods
Here we revisited our analysis using a more recent version of GENCODE and carried out a similar in depth investigation into the RefSeq and UniProtKB reference proteomes. We used a range of data sources to test whether genes were potentially not coding, including features from four databases, transcript and antibody evidence from the Human Protein Atlas, MS evidence from PeptideAtlas, copy number variations, and genetic variation from the 1000 Genomes Project.

Results
We tagged 2,278 GENCODE genes as potentially non-coding based on unusual and non-coding features. These genes had poor transcript evidence and little evidence of protein expression with antibodies and proteomics. Human genetic variation data suggests these genes are under close to neutral selection pressures. We find the same pattern for genes not classified as coding by all three manual annotation, one in eight of all genes classified as coding.

Conclusions
We find that a relatively large proportion of genes classifed as coding across the human reference genomes may not code for proteins. We predict that the number of protein coding genes may end up being fewer than 19,000.

Keywords
Genetic variation, coding genes, reference genomes

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Stratification biomarkers in personalised medicine for osteoarthritis: results from the MOVES study.

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Stratification biomarkers in personalised medicine for osteoarthritis: results from the MOVES study.
Background: The aim of this study was to identify predictive protein biomarkers useful to stratify osteoarthritis (OA) patients into responders and non-responders, either to Droglican® (glucosamine hydrochloride+chondroitin sulfate) or the COX-2 selective nonsteroidal anti-inflammatory drug Celecoxib, in order to optimize therapeutic outcomes in OA.

Methods: A shotgun proteomic analysis was performed on sera from 80 patients enrolled in the Multicentre Osteoarthritis interVEntion trial with Sysadoa (MOVES), employing the iTRAQ labelling technique followed by LC-MALDI-MS/MS analysis. In order to verify the specificity and sensitivity of a 6 proteins panel, we analyzed the whole cohort at baseline (n=500) employing conventional enzyme-linked immunosorbent assays (ELISAs).

Results: In the discovery phase, the proteomic screening led to the identification of a panel of six putative predictive protein biomarkers specific for the Droglican-responders group. In the verification phase, serum levels of APOA2, APOA4, APOH, C4BPa, ITIH1 and ORM2 were measured by ELISA assays. Non parametric analysis showed decreased levels of ORM2 at baseline in responders to Droglican compared to non-responders (76,11±53,25 vs 104,25±84,93 ug/mL; n=171 vs 46; p=0,047), meanwhile the values for APOA2 appeared statistically increased in responders compared to non-responders (79,95±58,53 vs 66,05±46,49 ug/mL; n=129 vs 112; p=0,028). Patients with lower levels of ORM2 and/or higher level of APOA2 showed a markedly better response to pharmacotherapy. Statistical interactions between ORM2 and APOA2 levels and OA radiologic grade were also detected (p=0,048 and p=0,002 respectively). No statistically significant differences were found for the other four proteins.

Conclusions: Our results show that ORM2 and APOA2 levels significantly correlate with patients response to Droglican suggesting the possibility of their use in predictive assays in order to optimize therapeutic outcomes in OA. Validation studies in different cohorts are needed to determine an optimal cut-off point for these biomarkers.

Keywords: osteoarthritis, predictive biomarkers, iTRAQ, ELISA.

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Towards the standardization of mitochondrial proteomics: the Italian mt-HPP initiative

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Background: The mitochondrial Italian Human Proteome Project initiative (mt-HPP) is a HUPO initiative led by the Italian Proteomics Association (ItPA), focused on human mitochondrial proteins. The main goal of this effort is to obtain robust information about the integrative role of proteins acting at the mitochondrial level, considering those encoded by both mitochondrial (mt-DNA) and nuclear DNA.
Methods: Fifteen labs were involved. Ten ECACC cell lines were selected (BJ, SH-SY5Y, U2OS, MDA-MB-231, NCI-H28, Hek293, HUVEC, THP1, HepG2, HeLa). Three methods for mitochondrial isolation were chosen (differential centrifugation, sucrose gradient separation, a commercial kit based on surfactants). Samples prepared with two different methods from the same cell line reached the same MS lab. Seven different mass spectrometers were used (Bruker Maxis HD and Impact HD, Waters Synapt G2si, Thermo Fusion, Velos ETD and LTQ-Orbitrap-XL, Sciex TripleTOF 5600+) and all the raw data were sent to the same center for data analysis. Proteins were selected if observed in at least two out of three biological replicates (two technical replicates each) with a ratio (-10logp/MW)>0.0005, and further annotated as mitochondrial proteins (Mitocarta and IMPI).
Results: Two lists of identified mitochondrial proteins (two different enrichment protocols) were generated for each cell line. After filtering as described above, the number of identified proteins varied from about 300 to 700. The recovery of mitochondrial proteins was similar in all preparations. Nevertheless, the mapping of proteins on the mitochondrial functional proteome network (Fasano et al., EuPA Open Proteomics 2016) revealed differential enrichments of mitochondria-associated proteins.
Conclusions: The comparison of the results revealed which preparations were more suitable for particular cellular models or for an increased recovery of mitochondrial associated proteins that may constitute an important component when studying mitochondrial biology.
Keywords: Mitochondria, standardization, isolation protocol, mt-HPP

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Towards comprehensive signaling pathway monitoring using advanced PRM methods

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Background

Targeted mass spectrometry quantification based on high-resolution and accurate-mass (HRAM) parallel reaction monitoring (PRM) measurements is increasingly adopted by the proteomics community. Combined with advanced acquisition schemes, e.g., internal standard triggered-PRM (IS-PRM), the technique benefits from further improvement in experiment scale and data quality [1]. Here the performance of PRM has been revisited to evaluate the advantages enabled by the latest technology developments. The new state-of-the-art methods were applied to the wide targeted profiling of signaling pathways, demonstrating an unprecedented combination of coverage depth and breadth.

Methods

The analyses were performed on Thermo Scientific™ Q Exactive™ mass spectrometers (QExactive HF and modified QExactive) operated with a broad range of PRM methods, including several variants of IS-PRM (some of which using instrument programming interface).

Results

The modified quadrupole-Orbitrap mass spectrometer offers several hardware and acquisition method features, beneficial to PRM analyses. Among advanced methods, the IS-PRM technique has been adapted to leverage latest technology developments. In addition, a new variant of IS-PRM method dedicated to very large-scale experiment was developed, relying on the acquisition of a few to single spectrum to measure simultaneously each pair of IS and endogenous peptides. The new features and methods were evaluated and applied to the monitoring of signaling pathways (including AKT/mTOR and RAS pathways) in various human cancer cell lines, including the measurement of peptide dilution series (40 peptides) and very large screening experiments (600-700 peptides). The improved scanning rate in the watch mode of IS-PRM and the new variant of IS-PRM were decisive to the broad coverage of signaling pathway achieved.

Conclusion

Unprecedented depth and breadth of coverage in signaling pathway monitoring was achieved through advanced PRM methods and modified QExactive instrument.

Keywords

Targeted proteomics; Parallel reaction monitoring; signaling pathway monitoring, human cancer cell lines

[1] Gallien , Kim, and Domon; MCP, 2015

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Tissue specific secretomes, the hidden treasure for identification of disease related marker proteins.

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Background

Modulations of tissue-specific secretome profiles, triggered by obesity and sedentary lifestyle, or influenced by physical activity are supposed to play a crucial role in the development, prevention and therapy of metabolic diseases including type 2 diabetes.

Methods

The most crucial step to get closer to valid and novel identifications is utilizing an adequate sample processing. We integrate different proteomic profiling techniques (Gel-based and gel-free MS approaches, Multiplex Immunoassays etc.) for a comprehensive characterization of the complex tissue secretomes. Preparation according to standard operation procedures combined with close quality control are irreplaceable in order to achieve high quality samples suitable for secretome analysis.

Results

We established an analysis platform to profile tissue specific secretomes for adipose tissue, skeletal muscle, hepatocytes and islets. These comprehensive protein maps, available under Diabesityprot.org, will help to achieve a deeper understanding for the complex and dynamic interplay of proteins involved in the communication between different tissues and its alteration in disease pathophysiology.

Conclusions

Tissue specific secretome profiling, provide a treasure trove of novel disease related marker proteins, which potentially can be used for diagnostic or therapy purpose of multifactorial metabolic disorders.

Keywords

tissue secretomes, targeted and non-targeted proteome profiling, biomarker discovery

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Efficient modelling of signalling networks derived from mass spectrometry phosphoproteomic data using Integer Linear Programming

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Background: Mass spectrometry analysis of proteome modifications offers huge potential for the studying of how therapeutic kinases inhibitors affect the signalling mechanisms inside the cell.

Methods: We have recently proposed PHONEMeS, a method which handles high-content shotgun phospho-proteomics data upon perturbation, tailored to the nature of this data, in particular to the under-sampling of the phosphoproteome. PHONEMeS puts the data in a context of kinase/phosphatase to substrate knowledge from which we derive and train logic models that can be used to gain insights on how cellular processes can be regulated after drug perturbation and predict signalling mechanisms by observing how this perturbation propagates from the inhibited target kinases toward measured phosphosites. Mathematically PHONEMeS solves an optimisation problem that in our original implementation was very demanding computationally.

Results: We have reformulated PHONEMeS as an Integer Linear Program (ILP) , that is orders of magnitude more efficient than the original one, enabling a larger and more sophisticated analysis. We illustrate the value of the new approach on various data sets of medical relevance where we shed light on signalling mechanisms and drug's mode of action.

Conclusions: The ILP formulation of PHONEMeS provides an efficient way to study the effects of kinase inhibitors and gain insights on the modulation of cell signalling mechanisms upon drug perturbation.

Keywords: Shotgun MS, Post-translational modifications, Signalling networks, Modelling, Integer Linear Programming

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Interactome analysis of the RLTPR protein reveals its essential role in T cells

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Background

The RLTPR cytosolic protein, also known as CARMIL2, is essential for CD28 co-stimulatory functions in mouse T lymphocytes, but its importance in human T cells and mode of action remain elusive. The RLTPR interactome identification can help to understand the mechanism of action of RLTPR during CD28 co-stimulation in mouse and human T cells.

Methods

We developed mice that bear a genetic tag (one-STrEP-tag; OST) allowing affinity purification - mass spectrometry analysis (LTQ Velos Orbitrap) of RLTPR interactome in primary T cells. Thymocytes containing high levels of RLTPR were used to determine RLTPR interactome at different time of stimulation using pervanadate, a potent chemical component mimicking early molecular events induced during the T cell activation. Three independent biological experiments, each involving five different conditions corresponding to no stimulation and to four time points after stimulation were analysed by AP-MS.

Results

We showed that known molecular components of the CD28 signaling pathway, such as CD28 itself, GRB2, VAV1 and CARMA1, physically associate with RLTPR after activation. Additionally, we also identified uncharacterized molecular elements potentially relevant to elucidate the RLTPR CD28-dependent co-stimulatory function. These results suggested that RLTPR acts as a scaffold protein, bridging CD28 to downstream effectors allowing the specific receptor transduction in T cells.

Conclusions

19 proteins were determined as the RLTPR interactome of thymocytes. Among these proteins, we identified a large spectrum of functional activities, including phosphatases, adaptors and proteins involved in the actin dynamic assembly. Whereas some of these proteins are constitutively associated with RLTPR, a majority of interactors showed a dynamic interaction with the bait after T cell activation.

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The human neutrophil: improved proteome coverage and clinical insights

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Background

The human neutrophil proteome is relatively poorly-researched. Sub-Saharan Africa is responsible for ~80% of global HIV-associated TB, and improved ART access has increased TB-IRIS incidence during co-treatment. TB-IRIS produces significant morbidity and mortality, but immunopathogenesis is incompletely understood. Preliminary data implicate neutrophils in TB-IRIS pathogenesis.

Methods

We have assembled a clinical cohort consisting of ART-naïve patients on treatment for HIV-associated TB (and at risk for TB-IRIS). Phlebotomy was performed just prior to, and at the typical time of TB-IRIS onset after, ART initiation. One-hundred-ninety-six extracted neutrophil proteomes are undergoing mass spectrometry-based differential profiling in order to generate biological hypotheses for orthogonal validation. This sample size provides >95% power to detect a two-fold change in protein expression with 99% probability, and will facilitate various inter-class comparisons and sub-group analyses (also incorporating a high-quality healthy volunteer neutrophil dataset generated in-house). Various methodological and bioinformatic strategies have been employed to maximise neutrophil proteome coverage and dataset utility.

Results

Healthy volunteers in combination with our unique clinical cohort represent an opportunity to study the neutrophil proteome under uniquely varied conditions. Neutrophil isolation yield (cells per sample) and purity exceed 19x106 and 90%, respectively. Protein extraction yield exceeds 60µg per 106 cells. Liquid chromatography-coupled mass spectrometry, incorporating gradient optimisation but no pre-fractionation, identifies over 2200 neutrophil proteins. Preliminary data suggest significant differences in neutrophil protein expression exist between comparison classes. Many of the observed proteins belong to classes over-represented by the remaining 'missing' human proteins (e.g. beta-defensins, cadherins, leucine-rich repeat-containing proteins, solute carrier family members, and zinc finger-, coiled-coil domain-, and ankyrin-repeat domain-containing proteins).

Conclusions

Our approach may identify neutrophil peptides or proteins not previously observed, and a better understanding the immunological pathways underlying TB-IRIS pathogenesis will facilitate improved prediction, prevention, diagnosis, and management.

Keywords

human
neutrophil
mass spectrometry
clinical proteomics
bioinformatics

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Impairment of Protein Mitochondrial Quality Control (PMQC) in Amyotrophic Lateral Sclerosis

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Background. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by selective and progressive loss of motorneurons. Several etiologic factors are involved in ALS pathogenesis, however it is well accepted that a severe mitochondrial dysfunction leads to an unavoidable neuronal death. Despite this, there is no certainty on the intimate mechanisms sustaining the progressive neuronal demise. Therefore, the aim of this study is to decipher the processes and the molecular mechanisms that contribute to mitochondrial stress linked to ALS-related neurodegeneration.

Methods. We chose as representative ALS biological model SOD1-G93A transgenic mice at different developmental/disease stages. Proteomics analysis of mitochondrial enriched fractions derived from spinal cord tissues was carried out to evaluate the adaptation of ALS mitochondrial proteome. Differential protein expression was evaluated by shotgun proteomics analysis based on nLC-HDMSe. Moreover, a targeted proteomics analysis of key ALS proteins has been performed in mitochondria derived from motor cortex, brainstem and cerebellum tissues, focusing on persulfide containing proteins and peptides.

Results. Proteomic analysis revealed interesting mitochondrial proteins differentially expressed in SOD1-G93A mice spinal cord compared to wt. Among these, subunits of cytochrome complexes, heat shock proteins (e.g. HSP10, 60 and 70), protein disulfide isomerase, GAPDH. Their expression has been also studied in mitochondria derived from other neuronal tissues.

Conclusion. Adaptation of ALS mitochondria proteome repertoire has revealed an impairment of mitochondrial quality control, system addressed to maintain mitochondrial homeostasis of via their regulated biogenesis and protein degradation. Moreover, most of differentially expressed proteins show a common element, they have persulfide sites and are H_2S targets. Therefore, we retain that hydrogen sulphide (H_2S) further distress and amplify an already compromised mitochondrial function in the vulnerable ALS motor neurons.

Keywords: ALS- H_2S-mitochondria-proteomics-persulphydration

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Applying a targeted Proteoform Profiling method for neurological disorder biomarker discovery

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Background

The low abundance Tau protein is routinely used as a molecular biomarker for Alzheimer's disease (AD) through the quantification of total Tau and of the phosphorylated Tau peptide. As this approach has specificity and accuracy, a targeted proteomics method has been developed to enable the direct quantification of the six Tau isoforms in CerebroSpinal Fluid (CSF). Discriminating the various Tau isoforms requires good sequence coverage ; combined with the low abundance of the targeted isoforms and the overall CSF complexity, this constitutes an analytical challenge and might reduce the viability of the approach in a clinical environment.
As Top-Down approaches reveal isoform distribution patterns while removing the hassle and variability of the digestion step in bottom-up methods, we are developing a targeted top-down proteomics approach. The purpose of this approach will be to deliver information on the relative abundance of the various Tau isoforms while being compatible with the requirements of the routine clinical laboratory.

Methods

The Tau protein isoforms are enriched from CSF samples by using an automated immunoprecipitation (IP) procedure. A proteoform profiling approach that uses a state-of-the-art UHR-Q-TOF is then used to generate the isoform profiles.

Results

After optimization of the LC-MS profiling method to increase the detectability of the various Tau isoforms, the method has been used to optimize the IP procedure. A Tau standard spiked in CSF has been used to verify that the process was not altering the isoforms distribution ratio. The proteoforms of the tau ladder, ranging from 36 to 45 Kda, are easily separated by the method and the reproducibility of the isoform ratio determination has been evaluated, before evaluating the method on endogenous Tau isoforms in CSF
In this communication, we are presenting the reproducibility test results as well as the first results obtained from patient samples.

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Host macrophage modulation of cell death and related pathways during early MSSA and MRSA infection

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Background
MRSA is one of the major human and veterinary pathogen responsible of multiple serious infections and outbreaks Methicillin-resistant Staphylococcus aureus (MRSA) resistance is due to the acquisition by Methicillin-susceptible (MSSA) strains of the mobile gene segment mec (SCCmec). Moreover, few investigations have been made on MSSA despite the importance in resistance acquisition and as source of infection. The aim of this project is the investigation of the host proteome modulation during strain-specific infection at different time-points using a quantitative label-free approach.
Methods
Protein expression changes at 1h and 2h in MRSA and MSSA infected THP1 cells have been detected using a label free approach. Three biological replicates has been analysed in quadruplicate using a SYNAPT G2-Si (Waters) applying the MSE data acquisition strategy coupled to IPA (Qiagen) bioinformatics analysis
Results.
Label free MSE led to identification of 110 and 157 differentially expressed macrophage proteins ($p<0.05$) after 1h and 2h of infection, respectively. Of 157 macrophage proteins 103 of them have been found exclusively downregulated in mrsa infection after 2h and 33 and 21 respectively down and up regulated in MSSA infected THP1 after 2h. Ingenuity pathway analysis highlighted several canonical pathways and bio-functions differentially regulated during the infection stage. In particular, apoptosis seems to be inhibited at 1h of infection in MSSA infected THP-1 compared to the other time points and to MRSA. Moreover, on the basis of the host protein modulation there is a possible evidence of early pathogen exit from host cells in the MSSA infection.
Conclusions
Proteomic data obtained in this work could provide new knowledge about the early molecular response of the host during the infection with specific MRSA and MSSA strains.

Keywords: MSSA, MRSA, THP1, MSE, infection, cell death

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Global Proteomics of Tauopathy Brains to Identify Tauopathy-differentiating Disease Pathways

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Background: Neurodegenerative diseases are considered to be a major threat to current healthcare systems due to the increasing number of patients and the lack of any meaningful therapeutic approaches. Tauopathies, including its most common form – Alzheimer's disease (AD) account for the majority of the neurodegenerative diseases. Although various different tauopathies are known, the exact diagnosis is often only possible post-mortem and there are no therapies currently available. To better understand the different tauopathies and to identify tauopathy-specific pathways of dysregulation, we comprehensively and quantitatively mapped proteins in the angular gyrus region of tauopathy-related post-mortem brain samples in comparison to healthy age-matched controls.

Methods: The proteomes of 30 post-mortem angular gyrus tissue samples (10 AD, 10 Progressive Supranuclear Palsy (PSP) and 10 age-matched non-demented control brains) were extensively analyzed using isobaric labeling-based quantitative proteomics strategies. In addition, we analyzed the sarkosyl-insoluble and -soluble proteome fractions from brains (angular gyrus region) of 125+ patients diagnosed with tauopathies including AD, PSP, Pick's Disease, Corticobasal Degeneration and non-demented age-matched controls.

Results: The labeling-based quantitative proteomic analysis of 30 human brain tissue samples resulted in the identification ~10,000 proteins, providing a comprehensive map of the angular gyrus proteome and the tauopathy-associated changes. This map identified numerous druggable pathways that showed dysregulation in a tauopathy specific manner. Similarly, the mapping of the sarkosyl-insoluble fraction provided unprecedented insights into the protein composition of the Tau aggregates.

Conclusions:Distinct proteins and molecular pathways show statistically significant tauopathy specific up-/down-regulation in the different proteomes. Amongst the top 20 proteins enriched in the AD were the well characterized AD-associated proteins such amyloid beta-4, serum amyloid-P and S100A-4, validating our analytical strategy. Proteins changing in AD were predominately associated with RNA processing and splicing, immune system and protein phosphorylation pinpointing to novel pathways that may be involved in AD.

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Proteomic characterization of neuromelanin granules isolated from human substantia nigra by laser-microdissection

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Neuromelanin (NM) is a complex polymer pigment found primarily in the dopaminergic neurons of human substantia nigra. Neuromelanin pigment is stored in granules including a protein matrix and lipid droplets. NM is of special interest because in Parkinson's disease and related disorders pallor of the substantia nigra pars compacta (SN) can be seen, due to the depletion of dopaminergic neurons containing NM. Thus we hypothesise NM to be involved in the pathogenesis of respective diseases. To clarify the exact function of neuromelanin granules in humans, their enrichment and indepth characterization from human substantia nigra is necessary. Our previously published global proteome studies of neuromelanin granules in human substantia nigra required high tissue amounts [1, 2]. Due to the limited availability of human brain tissue we established a new method based on laser-microdissection combined with mass spectrometry for the isolation and analysis of neuromelanin granules [3]. With this method it is possible for the first time to isolate a sufficient amount of neuromelanin granules for global proteomics analysis from ten 10 µm tissue sections. In total 1,000 proteins were identified associated with neuromelanin granules. More than 68% of those proteins were also identified in previously performed studies. Our results confirm and further extend previously described findings, supporting the connection of neuromelanin granules to iron homeostasis and lysosomes or endosomes. Hence, this method is suitable for the donor specific enrichment and proteomic analysis of neuromelanin granules. First comparative proteome studies revealed substantial differences in the NM proteome pattern from control and diseased individuals.

[1] Tribl F et al., MCP 2005
[2] Plum et al., J Proteomics 2013
[3] Plum et al., Sci Rep 2016

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ABSOLUTE VENOMICS: ABSOLUTE QUANTIFICATION OF INTACT VENOM PROTEINS THROUGH ELEMENTAL MASS SPECTROMETRY

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Background
Snakebite envenoming has become a serious, yet neglected, global public health issue that affects the most impoverished and geopolitically disadvantaged rural communities of Africa, Asia, Latin America and parts of Oceania [http://www.snakebiteinitiative.org]. Unveiling how natural selection has molded the temporal and spatial patterns of venom phenotypic variability we observe today is also of fundamental importance to understand the molecular basis of envenoming, a prerequisite for developing therapeutic strategies to counteract its tragic consequences.
Methods
We report the application of a hybrid element and molecular MS configuration for the parallel absolute quantification of µHPLC-separated intact sulfur-containing venom proteins, via ICP triple quadrupole MS and 32S/34S isotope dilution analysis, and identification by ESI-QToF-MS of the toxins of the medically important snakes.
Results
The suitability of the hybrid LC-MS configuration to simultaneously identify and absolute quantify (in µmol protein/g venom) sulfur-containing venom proteins was validaded using the venoms of N. nigricollis, M. ikaheka, and P. papuanus.
Conclusions
The combined application of element and molecular MS opens new possibilities for proteomic analysis in general, and phosphoproteomics and venomics in particular. The capability of the hybrid LC-MS configuration µHPLC-ESI-QToF/ICP-QQQ with on-line isotope dilution analysis to quantify full-length proteins offers the attractive possibility of performing the parallel absolute quantification and the identification of the (venom) proteins by means of top-down approaches. However, a major challenge ahead is to develop a robust platform to achieve pre-MS separation of each and every protein species in the sample.

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Terminal Amine Isotopic Labeling of Substrates (TAILS) approach to study mitochondrial proteases in Parkinson's disease

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Background: Mitochondrial dysfunction appears to have a major pathogenetic role in Parkinson's disease (PD). The surveillance of protein quality control within mitochondria is orchestrated by a highly conserved proteolytic system, whose impairment may contribute to the onset of PD. Using a cellular model of impaired dopamine (DA) homeostasis, which mimics a likely early event in PD pathogenesis, it has been recently proposed that DA aberrantly activates mitochondrial proteases.

Methods: The aim of the present work was the identification of candidate dopamine-activated mitochondrial proteases involved in PD pathogenesis. To this end, mitochondrial protein fractions have been isolated from SH-SY5Y cells treated or not with DA, and the N-terminome was enriched and analyzed by a newly developed positional proteomics approach named TAILS (Terminal Amine Isotopic Labeling of Substrates).

Results: A detailed analysis of the N-terminome of dopamine-treated SH-SY5Y cells has been performed, thus leading to the identification of dopamine-induced proteolytic cleavages in mitochondrial proteins.

Conclusions: The sequence of the DA-induced proteolytic fragments will lead to the identification of candidate mitochondrial proteases through a bioinformatics approach. The identified enzymes would represent novel drug targets or diagnostic biomarkers for PD.

Keywords: Mitochondrial proteases, Parkinson's disease, Quantitative proteomics, N-terminome analysis.

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Affinity proteomics identifies proteins associated with asthma severity

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Background
Asthma and chronic obstructive pulmonary disease (COPD) are major respiratory diseases where there is a need for biomarkers that can identify disease subgroups. We applied an affinity proteomics approach, performing protein profiling of 895 samples from 675 individuals with asthma of different severities, COPD and healthy controls.

Methods
Protein levels were analysed using antibody suspension bead arrays. Selected targets included proteins of interest to asthma, COPD, lung and inflammation in general, resulting in 180 proteins detected by 377 antibodies. The bead array was used to screen plasma samples from the U-BIOPRED study (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes): mild to moderate asthma, severe asthma, severe asthma and current or ex-smoker, and healthy controls. Samples were also analysed using the SOMAscan assay. The bead array was then used to profile samples from the BIOAIR study (Longitudinal Assessment of Clinical Course and Biomarkers in Severe Chronic Airways Disease): mild to moderate asthma, severe asthma or COPD. BIOAIR patients took part in a placebo-controlled two week oral steroid intervention trial.

Results
The U-BIOPRED study revealed most significant differences between severe asthma and mild asthma or controls. In the intervention study, the majority of protein levels decreased after steroid treatment in all groups. The number of significantly altered proteins was larger in the mild asthma group followed by severe asthma and COPD. A number of proteins were verified in both cohorts and with the SOMAscan assay. These included C9, CHIT1 and LEP, with higher levels in severe asthma compared to mild and controls.

Conclusions
Protein profiling in two independent cohorts revealed protein differences potentially related to the severity of asthma. Further analysis will focus on connecting the results to clinical information with the aim to reveal new subgroups for increased understanding and better disease management.

Keywords: antibody arrays, asthma, plasma

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TISSUE PROTEOMIC ALTERATIONS OF COLON ADENOCARCINOMA

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Introduction and objectives: Adenocarcinomas are the cancers originating from the gland forming cells of the colon and rectal lining and are known to be the most common type of colorectal cancer. In this study, we used proteomics approach with an aim to identify protein biomarkers which can aid in early detection of colon adenocarcinomas to be precise.

Methods: Proteins from tumor tissue of colon adenocarcinoma subjects (n=11) and their matched controls were subjected to 4-plex iTRAQ labelling followed by off-gel fractionation prior to LC-MS/MS run. The proteins identified using either Spectrum Mill and/or Trans Proteome Pipeline were subjected to DAVID and the proteins common between the two analyses were compared with the data from CPTAC portal. The expression level of few of the shortlisted panel of proteins was validated using MRM approach.

Results and Discussion: A list of 285 unique proteins was identified to be significantly dysregulated in colon adenocarcinoma as compared to its matched controls using either SM or TPP. These proteins were found to be involved in glycolysis, pentose phosphate pathway, biosynthesis of amino acids, protein processing, spliceosome, proteosome, focal adhesion and proteoglycans in cancer. 94 of the 285 proteins were identified by both- SM and TPP. 34 of these 94 proteins were found to be dysregulated with same trend as that in data reported on CPTAC portal and 17 of these 34 proteins were not identified/not altered with same trend or not significantly altered in either grades of glioma and meningioma. 7 of these 17 proteins were validated using MRM approach.

Conclusion: The proteins identified from this study could be investigated further to unravel the potential of these proteins to be considered as biomarkers for early detection of colon adenocarcinoma.

Keywords
Colon Adenocarcinoma, Tissue, Biomarker, iTRAQ, MRM

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Identification of cartilage endogenous peptides differentially released in the serum of osteoarthritic and healthy patients

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Background

Osteoarthritis is the most prevalent musculoskeletal condition. This disease is mainly characterized by the degradation of articular cartilage, but lacks from efficient tools for its precise diagnosis and monitoring. Previous peptidomic experiments showed a number of endogenous peptides differentially released from human articular osteoarthritic damaged cartilage, compared to healthy controls. In the present work, we aimed to monitor and compare the presence of this panel of peptides also in synovial fluid (SF) and serum samples from osteoarthritis (OA) patients and healthy donors (N).

Methods

The enrichment of endogenous peptides in serum and SF samples was accomplished by acetonitrile precipitation followed by ultrafiltration. After peptide extraction, the different peptide profiles of these samples were analyzed by shotgun mass spectrometry using a nLC-LTQ-Orbitrap. MRM methods were developed on a 5500 QTRAP using Skyline software.

Results

Sera from 3 hip OA patients, 3 knee OA patients and 3 healthy controls was analyzed by nLC-MS/MS, following a label-free strategy. The shotgun analysis led to the identification of more than 500 endogenous peptides, allowing the characterization of differential profiles between the three types of samples. Among these, 161 peptides corresponding to 33 different proteins were present also in SF and cartilage secretomes. These include endogenous peptides from cartilage-characteristic proteins such as Matrix Gla Protein, Extracellular Matrix Protein 1 or Fibronectin. A targeted identification method based on Multiple Reaction Monitoring (MRM)-mass spectrometry was developed to validate the previous analysis by nLC-MS/MS and confirm the identifications with heavy-labeled synthetic peptides in a larger cohort.

Conclusions

Altogether, this work provides a panel of endogenous peptides that may be useful for the molecular diagnosis of OA. The qualification of the biomarker value of this panel for patient stratification, disease prognosis and/or therapy monitoring is currently ongoing using the developed MRM method.

Keywords
Osteoarthritis, biomarker, peptidomics, serum, synovial fluid.

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Social defeat stress: A plasma proteomic study to identify stress susceptible-associated and resilience-associated biomarkers

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Background: According to the social defeat (SD) hypothesis, long-term exposure to the experience of SD may lead to sensitization of the mesolimbic dopamine (DA) system and thereby increase the risk for schizophrenia. The hypothesis posits that SD (i.e., the negative experience of being excluded from the majority group) is the common denominator of 5 major schizophrenia risk factors: urban upbringing, migration, childhood trauma, low intelligence, and drug abuse.

Methods: Serum was collected from mice that underwent social defeat experiments and were identified as control, susceptible, or resilient. The blood was collected, allowed to clot at room temperature and then spun at 4°C. Plasma was depleted of the three most abundant proteins, i.e. albumin, IgG, transferrin. Unbiased shotgun proteomics incorporating label-free quantitation was used to identify differentially expressed proteins.

Results: 170 proteins passed the initial quality check i.e. proteins were identified in 80% of samples. Repeated measures analysis within the susceptible group comparing day 0 and 30 revealed 49 proteins to be significantly differentially expressed, of which 19 were FDR positive. Testing the control versus susceptible group, both at day 30, showed 23 significant proteins, but none were FDR positive. Fourteen of the significant ($p<0.05$) proteins in this measurement overlapped with the repeated measures susceptible group. Repeated measurements in control mice only, at day 0 and 30, found 38 proteins to be significantly expressed, seven of them FDR positive. This indicates an effect of the behavioural testing of the animals and also points to an age effect. These proteins were excluded leaving a final list of 29 proteins at $p<0.05$ and 15 proteins that are FDR positive.

Conclusions: We found that proteins involved in the Complement and Coagulation system are dysregulated and will be using a human stress model to validate these findings.

Keywords: stress, susceptibility, resilience, biomarker

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Addressing the challenges of quantitative myelin proteomics

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Background
Myelin, a plasma membrane specialization of glial cells, facilitates rapid nerve signaling and thus normal motor, sensory and cognitive functions. Proteomic analysis of myelin is challenging because of its high content of lipids and membrane proteins, and its unfavorable dynamic range. Thus, technical advances are required, e.g. to approach failing glia-axonal interactions in neurological disorders.

Methods
To improve the accessibility of myelin proteins and to minimize digestion variability, we developed a FASP-based in-solution digestion procedure and implemented it into a robotic liquid handling workstation (Tecan Freedom EVO 150). Data were acquired on a QTOF LC-MS-system (Waters Synapt G2-S) using data-independent acquisition (DIA) strategies without (MSe) and with (HDMSe) ion mobility separation. Label-free protein quantification was performed with the IsoQuant software (www.isoquant.net).

Results
We used label-free protein quantification to compare CNS myelin from wildtype mice (CnpWT/WT) with myelin from mutant mice heterozygous for the CNP gene (CnpWT/null). In three independent experiments, each comprising 24 LC-MS runs (three biological replicates per condition, duplicate digestion and injection), we quantified ~500 (MSe) and ~1000 (HDMSe) proteins, of which ~80% were present in all three experiments. By MSe, the ratio of the abundance of CNP CnpWT/WT:CnpWT/null was experimentally determined as 1.98:1 (CV < 10%) and thus exactly matched the ratio of 2:1 as expected from Cnp gene heterozygosity. By HDMSe, however, levels of highest-abundant myelin proteins including CNP were misquantified due to the compression of the dynamic range caused by ion mobility separation of peptides. To address this issue, we propose a novel data acquisition method, which provides a compromise between proteome depth and the enhanced dynamic range required for quantitative myelin proteomics.

Conclusions
We have developed a robust workflow for accurate quantitative comparison of myelin proteomes, which will deepen our insights into myelin biology and pathology.

Keywords
myelin, glia-axonal support, FASP, automation, quantification

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Comparative proteomic analysis of mouse kidney with systemic lupus erythematosus

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Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease affecting different organs including, brain, heart, joints, skin and kidneys. Lupus nephritis (LN), an inflammation of the kidney, is one of the most severe complications of SLE associated with a significant mortality and morbidity. In addition, nephritis classification criteria have poor prognostic value and thus they are not suited for tailoring therapy in individual cases. Therefore, identification of tissue-specific biomarkers for the prediction of kidney failure caused by SLE, will enable the development and application of targeted clinical therapies. Herein, using mass spectrometry (MS)-based proteomics we examined the proteomic profiles in the lupus-prone B6.Sle123 mice and the respective B6 control mice, in order to gain insights into the mechanisms implicated in LN.

Methods

Mice used were of 12-, 20- and 36-weeks old, representing three different stages of SLE. Proteins were extracted from kidneys, purified, reduced, alkylated and digested by trypsin. Purified peptides were analysed on a Waters Synapt G2Si HDMS instrument operated in ion mobility mode using a UDMSe approach. Data were processed by the Progenesis QIp and functional annotation analysis performed using multiple bioinformatics resources.

Results

More than 3000 non-redundant proteins were identified in all sample. Comparison of proteomic profiles of disease and control mice kidneys, has identified several hundred proteins including immunoglobulins, histones and glycoproteins such as complement component C1q, were found to be altered over SLE disease progression. Further pathway analyses showed that the identified dysregulated proteins were involved in multiple pathways including, mitochondrial electron transport chain, cell redox homeostasis and actin-binding, which are known to be associated with autoimmune diseases.

Conclusions

Using MS-based proteomic analyses of mice kidneys with SLE at different stages, we identified a number of proteins that are implicated in LN development and hence, they can serve as potential biomarkers of LN.

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Matrisome landscape of Head and neck squamous cell carcinoma (HNSCC) -associated fibroblasts

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Background
HNSCC is the sixth most common malignancy reported worldwide with a 5-year survival rate of 50%, largely due to recurrent disease and locoregional spread following treatment failure. Malignant progression of these tumors is controlled by functional interplay between tumor cells and their pro-tumoural tissue environment thus a thorough understanding of these interactions is required for the development of effective therapies. The extracellular matrix (ECM) is a key component of the tumor microenvironment that shapes the tumor tissue and actively participates in tumor progression through complex signaling networks, not fully understood.

Methods
De-cellularised ECM was prepared from normal immortalized human skin fibroblasts (Telomerase-immortalized fibroblasts (TIFs)) and from early passage HSNCC-derived fibroblasts (CAFs) isolated from fresh tumours. Two independent biological experiments were analysed using mass spectrometer (MS).ECM extracts were reduced, alkylated, and proteins digested before injection on LC-MS/MS. The protein profile of CAFs and TIFs were compared using label-free quantification.

Aims and Results
We set out to provide a comprehensive data set of the human HSNCC-associated fibroblast matrisome. We focused on fibroblasts, as they represent the major matrix-producing cells of the tumour stroma. CAF and TIF matrisomes were compared to validate the possible use of TIF-derived matrix for functional studies. Cellular FN was among the major ECM glycoproteins produced by CAFs and TIFs. Other matrix glycoproteins include transforming growth factor-ß-induced (TGFBI), Emilin-1 and Tenascin C (TNC). Both matrisomes contained a similar set of core matrix components and matrisome-associated proteins with comparable relative abundance.

Conclusions
This dataset provides valuable insights into the adhesion-dependent events that take place in the microenvironment of these tumours.The ECM of human TIFs and CAFs display a similar composition, thus TIF-derived ECM can be utilized for functional studies, including ECM-tumour cell interactions and interactions between the ECM immune cells.

Keywords
Matrisome, HNSCC, FN, tandem MS/MS, Label-free

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Proteomic Analysis of the Impact of Metabolic Status and Genetic Predisposition on Oviduct Fluid Composition

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Background
Impaired fertility in dairy cows, also including early embryonic loss, has become an increasing issue over the last decades in correlation with a genetic selection for milk yield. Therefore, two animal models have been developed to study the influence of the genetic predisposition for fertility (genetic model) and the metabolic status (metabolic model) on the reproductive performance of dairy cattle.
Methods
Providing a crucial microenvironment for the gametes and the early embryo, oviduct fluid taken ipsilateral from ampulla and isthmic regions of both animal models has been analyzed in a holistic proteomic approach. The genetic merit model comprised Holstein heifers with high (HFH) and low (LFH) fertility index and heifers from the Montbéliarde breed (MBD), known to have a good genetic merit for reproduction. For the metabolic model, samples from maiden heifers (MH), lactating cows (Lact) and cows dried off after calving (Dry) were analyzed.
Results
Applying nano-LC-MS/MS analysis combined with a label free quantification approach, considerable proteome changes were detected comparing the different groups of animals. Of the 2240 proteins quantified, we found a total of 216 proteins to be significantly altered in abundance considering all pairwise comparisons. The largest number of differentially abundant proteins was detected between isthmic samples of MBD and LFH (52), MBD and HFH (47) followed by MH and Lact (19). Bioinformatic evaluation of the dataset comprising tools such as DAVID GO and gene set enrichment analysis showed the assignment of abundance altered proteins to the GO terms translation, immune response, cytoskeletal protein binding and enzyme inhibitor activity.
Conclusions
The dataset thus contributes to the assumption that immune processes play a role in early embryonic loss. Moreover, it comprises a pool of proteins that were so far not known to be involved in reduced reproductive performance of dairy cattle.
Keywords
Reproduction, oviduct fluid, bovine

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Post-translational Modification Profiles on Tau are Distinct for Alzheimer's Disease and Other Tauopathies

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Background: Immunotherapy approaches targeting Tau hold great promise for arresting or possibly reversing tauopathies. However, the exact nature of pathological Tau has not been thoroughly defined despite the fact that Tau is known to carry numerous post-translational modifications (PTMs). Our objective was to characterize pathological Tau in human diseased tissue and understand the differences of post-translationally modified Tau in health and disease.

Methods: We developed a novel mass spectrometry-based quantitative protein characterization platform that provides unprecedented accuracy for the PTM patterns on Tau and thus furthering the understanding the chemistry of Tau in the context of human pathology. The application of the platform to tau, named FLEXITau, enabled the comprehensive, unbiased, robust and highly sensitive identification and quantification of PTMs of Tau in in the context of 4 different tauopathies.

Results: Brain tissue specimens from more than 125 well-characterized dementia patients diagnosed with various tauopathies including Alzheimer's Disease (AD), Progressive Supranuclear Palsy, Corticobasal Dementia and Pick's Disease as well as age-matched controls were analyzed using FLEXITau. The study discovered and quantified 50 PTMs on Tau, the majority of which have not previously been described as occurring in vivo on human Tau. Furthermore, this analysis identified tauopathy specific modification patterns that i) allow for the objective differentiation of the various tauopathies, and ii) provides insight into tauopathy specific target candidates for immunotherapies.

Conclusions: The results of this study clearly demonstrate distinct profiles of post-translational modifications on Tau for each of these tauopathies relative to Tau in the control group. FLEXITau enables the identification of modifications distinct to each disease that will be useful for patient stratification and enables the identification of therapeutic epitopes that should be targeted in each disease.

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Systems pharmacology and quantitative proteomics for developing targeted triple therapy.

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Background

Targeted combination therapy describes an approach in medicine by which invading entities are specifically treated. Recently specific inhibitors blocking signaling pathways typically activated for solid tumor growth are being used to reduce tumor load. Despite success stories for the latter, it is still challenging to predict which drug combinations are most potent. Here we describe a novel approach combining systems pharmacology with targeted and quantitative proteomics for obtaining a list of candidate genes for subsequent short listing of triple target therapy.

Methods

We test our workflow on an early metastatic prostate cancer model LNCaP. First we generated a protein list of interest in prostate cancer (PLIPCa). Then we established tools for targeted proteomics approach and developed targeted proteomics assays. Lastly, we used systems pharmacology and quantified perturbed proteomes using both triple quadruple and high mass accuracy mass spectrometers.

Results

We systematically perturbed androgen receptor and PI3K-AKT-mTOR signaling cascade with single and double combinations quantifying PLIPCa using targeted proteomics. Most potent with the largest fold changes and highest statistical significance were dual pathway drug combinations. Surprisingly, there were several proteins strongly regulated as a function of perturbation. We hypothesized that a subgroup of these proteins were a common response of LNCaP cells to escape from treatment. We tested our hypothesis by inhibiting key proteins and found synergistic effects in the quantified proteome upon targeted triple treatment.

Conclusions

The presented pipeline delivers rapidly testable working hypothesis for triple targeted combination therapy. In the case of early metastatic prostate cancer cell line LNCaP, we find synergistic proteome changes upon triple targeted treatment. The extension of PLIPCa by proteins with large fold change and statistical significance is of potential interest for monitoring targeted therapy in prostate cancer.

Keywords

Targeted proteomics, systems pharmacology, combination therapy

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Standardising and harmonising multiple TripleTOF® systems for proteomics using a dedicated SWATH® Acquisition Performance Kit.

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Background
To get consistently high quality, reproducible quantitative proteomics data, proper controls are required. With this in mind a validated kit has been assembled to assess the performance of the TripleTOF® systems for IDA and SWATH® proteomics workflows. This kit is then used to monitor the performance of multiple systems at the Stoller Biomarker Centre used for biomarker discovery from clinically derived samples.
Methods
A set of 20 synthetic peptides of varied mass and retention time attributes has been developed for use as an infusion and LC-MS standard. LCMS performance is first monitored using this peptide mixture and then with a standard complex tryptic digest (human) is used to test the performance of the instrument in both IDA and SWATH® Acquisition modes. The total number of proteins and peptides quantified from the Swath® analyses are measured with CV's of <20% from 5 replicate runs.
Results
Eight 6600 TripleTOF® systems were benchmarked using the newly developed SWATH® Acquisition Performance Kit after the systems were installed and also after 6 months of operation. Using this kit we were able to show that on average 3050 proteins were identified from IDA runs and quantified from Swath® runs across all eight machines, with only a 5% variation in the number of proteins and 12% variation in the number of peptides quantified.

Conclusions
The Swath® performance of these eight instruments appears to be harmonized, so large cohorts of samples could be split over multiple instruments. 20 patient samples can be run per day per instrument so at full capacity the Stoller Centre could potentially analyse over 1000 clinical samples by microflow Swath® in one working week. The SWATH® Acquisition Performance Kit will be used to regularly measure instrument performance in these large scale biomarker discovery projects.
Keywords
Swath®, TripleTOF®, Biomarker Discovery.

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Peptidomics characterization of allergenic and non allergenic tropomyosin orthologs.

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Background
The cleavage and the digestion patterns of allergenic proteins play a key role in allergenicity. The transformation of food proteins starts with their denaturation by the acidic environment of the stomach. However, protein denaturation is not enough to completely remove allergenic properties of a protein, but it is necessary a complete enzymatic digestion. Whether the enzymatic digestion is not efficient, the persistence of bigger peptides can occur and put the basis for the development of the sensitization process.The hypothesis of the current work takes into consideration the probability that shrimp tropomyosin (TM) is not fully digested or presents a digestion pattern that generates some peptides that can be immunogenic.Therefore, the work plan designed, aims to study the cleavage pattern of: purified chicken TM, recombinant chicken TM, purified TM of Penaeus monodon (Pen m 1), recombinant TM of Penaeus monodon (rPen m 1) and recombinant TM of Crangon crangon (rCrac c 1).
Methods
One mg of each ortholog has been processed through simulated mouth, gastric and intestinal digestion. The sample was frozen to block the digestion and, after this step, concentrated and cleaned through protein precipitation. The protein pellet was processed for peptidomic analysis through 1D Tricine gel electrophoresis, 2D Tricine gel electrophoresis and mass spectrometry.
Results
Simulated gastric digestion pattern of shrimp TM highlighted the presence of a resistant band at an average MW of 25 kDa that could be involved in the immunogenic process.
Conclusions
This innovative approach (peptidomics study through 1D-2D Tricine/MS) could represent a milestone for the study of digestion patterns of allergenic proteins or for the study of allergenic potential of novel foods.

Keywords
Tropomyosin, digestomics, peptidomics, allergenic peptides

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Integrated Multi-Omics Characterization of Neuroblastoma

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Background
Neuroblastoma is a paediatric cancer characterised by very heterogeneous survival rates between risk groups. While certain mutational and transcriptional hallmarks of risk-stratification have been identified including e.g. MYCN amplification or chromosomal alterations, the mechanisms of some of these are features remain poorly understood.

Methods
We performed label-free shotgun proteomics on 34 patient biopsies. These data were combined with RNAseq-based transcriptomics data and 450K-array-based Copy-Number-Alteration (CNA) data from the same biopsies.

Results
We quantified more than 10.000 different protein groups and more than 45.000 different transcripts (including different isoforms and non-coding RNAs) across the 34 biopsies. As expected, transcript- and proteome-abundance were moderately correlated (median across biopsies rS=0.43). Comparing patients with MYCN amplification to those without, both transcriptomics and proteomics show upregulation of pre-rRNA complex and spliceosome. However, only the proteomics data show significant enrichments for Minichromosome maintenance protein complex, Bromodomain proteins and the BRD4–RFC complex in the list of proteins more abundant in patients with MYCN amplification. The importance of these observations is exemplified by ongoing clinical trials of Bromodomain inhibitors for neuroblastoma treatment, demonstrating the benefits of proteomics characterization.
Integration of CNA and proteomics data revealed a significant correlation between protein abundance of targets of transcription factor CREB and CNAs of chromosome 17q11.2-q12. This suggests a possible mechanism of action of chromosome 17q gain, which is a common amplification in neuroblastoma and associated with poor prognosis. Interestingly, there is no significant correlation between copy-number-changes of chromosome 2q33.3, where CREB is encoded, and chromosome 17q11.2-q12. Integration of proteomic-CNA-correlations with transcriptomic-CNA-correlations is currently ongoing.

Conclusions
Using proteomics analysis of patient biopsies, we identified important changes not identified by transcriptomics of the same samples, while integration of CNAs with proteomics unravel possible disease mechanism of CNAs.

Keywords
Neuroblastoma, Label-free quantification, Copy-Number-Alteration, Data Integration, Bioinformatics

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Comprehensive, Unbiased Proteomic Profiling of the Cell-Surface, Exosomal, and Secreted Proteomes of Senescent Cells

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Background
Cellular senescence is an irreversible arrest of cell division accompanied by secretion of cytokines, growth factors, and proteases collectively termed the senescence-associated secretory phenotype (SASP). The persistence of senescent cells and chronic SASP contribute to aging and numerous diseases by promoting chronic inflammation, tumorigenesis, and defective stem cell renewal. Selectively killing senescent cells is a promising strategy to combat these effects that has worked in mice; however, achieving this in humans will require senescent cell biomarkers. Here we perform proteomic characterization of surface, secreted, and exosome-associated proteins in senescent human fibroblasts.

Methods
To generate senescent (SEN) and control (CTL) cells, respectively, IMR90 primary human fibroblasts were x-ray or mock irradiated and cultured for 10 days. Then cells were placed in serum-free medium and collected after 24 hours to obtain secreted protein fractions (10x SEN, 10x CTL). Exosomes were collected from these fractions by high-speed centrifugation. Cell-surface proteins were isolated using Cell-Surface Capture, an in-vivo biotinylation/affinity enrichment approach. Data-independent acquisitions (DIA) were performed on a TripleTOF 6600.

Results
We identified >1000 secreted proteins with >500 significant differences from senescent versus control cells. These were almost exclusively increased in senescent cells and consisted of cytokines, growth factors, proteases, and clotting factors. In exosomes, over 400 proteins significantly changed (SEN vs CTL), including proteins involved in B-cell receptor signaling, integrin interactions, extracellular matrix organization, syndecan-1 signaling, notch signaling, and laminin interactions. Finally, 100 of the 305 surface proteins identified were differentially expressed in SEN and CTL groups. These mapped to pathways in cell death, translation, mRNA processing, and TGFbeta signaling.

Conclusions
Our comprehensive proteomics approach has revealed novel, differentially expressed proteins for the cell-surface, exosomes, and the secretome of senescent cells. We will further focus on validating biomarker candidates and assessing their utility for removal of human senescent cells in vivo.

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Quantification of PI3K/AKT/mTOR signalling pathway activity using immuno-MALDI mass spectrometry (iMALDI)

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Background

Colorectal cancer is one of the most common cancers in incidence and cancer-related deaths. The PI3K/AKT/mTOR pathway is commonly upregulated in colorectal cancer and the target of many anti-cancer therapies. Current patient stratification for targeted therapy is based mostly on genomic data, which has been shown to be insufficient.

The goal of this project was to develop immuno-MALDI mass spectrometry (iMALDI) assays to quantify the expression levels and phosphorylation levels of proteins in the PI3K/AKT/mTOR pathway. In combination with genomic data, these levels can be used to build a predictive model for a patient’s response to targeted therapy.

Methods

iMALDI is a method for protein quantification which combines antibody enrichment with detection by MALDI-TOF mass spectrometry. After enzymatic digestion of the sample, analyte-specific peptides, together with analogous stable-isotope labeled peptides, are enriched using antibodies immobilized to magnetic beads. The beads are then spotted onto a MALDI target and the target peptides are analyzed by MALDI-TOF MS.

Results

PI3K p110α, PTEN, and AKT I and II were selected as targets for assay development. Unique tryptic peptides containing the cancer-related phosphorylation sites were selected, confirmed experimentally, and used to raise polyclonal antibodies.
Quantification of AKT I and II expression-and-phosphorylation levels were achieved in various cancer cell lines, as well as in flash-frozen and formalin fixed tumour tissue samples, using 10 µg cell lysate.
Endogenous PI3K p110α and PTEN were detected in 25 µg MDA-MB-231 breast cancer cell lysate.

Conclusions

Methods for quantifying AKT I and II as well as PTEN and PI3K p110α were developed. The next steps of this project include combining the PI3K p110 α, PTEN, and AKT assays into a single multiplexed assay. Additionally, method validation will be performed using different cancer cell lysates and tumour tissue samples.

Keywords

Clinical Proteomics, Cell Signalling, Colorectal Cancer

MASP1, THBS1, GPLD1 and ApoA-IV are novel biomarkers associated with prediabetes: the KORA F4 study

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Individuals at a high risk of type 2 diabetes demonstrate moderate impairments in glucose metabolism years before the clinical manifestation of type 2 diabetes, a state called 'prediabetes'. In order to elucidate the pathophysiological processes leading to type 2 diabetes, we aimed to identify protein biomarkers associated with prediabetes.
In a proteomics study, we used targeted selected reaction monitoring (SRM) mass spectrometry (MS) to quantify 23 candidate proteins in the plasma of 439 randomly selected men and women aged 47-76 years from the population-based German KORA F4 study. Cross-sectional associations of protein levels with prediabetes (impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT)), type 2 diabetes, glucose levels in both the fasting state and 2 h after an OGTT, fasting insulin, and insulin resistance were investigated using regression models adjusted for technical covariables, age, sex, BMI, smoking, alcohol intake, physical inactivity, actual hypertension, triacylglycerol levels, total cholesterol/HDL-cholesterol ratio, and high-sensitivity C-reactive protein levels.
Mannan-binding lectin serine peptidase 1 (MASP1; OR per SD 1.77 [95% CI 1.26, 2.47]), thrombospondin 1 (THBS1; OR per SD 1.55 [95% CI 1.16, 2.07]) and glycosylphosphatidylinositol-specific phospholipase D1 (GPLD1; OR per SD 1.40 [95% CI 1.01, 1.94]) were positively associated with prediabetes, and apolipoprotein A-IV (ApoA-IV, 0.75 [95% CI 0.56, 1.00]) was inversely associated with prediabetes. MASP1 was positively associated with fasting and 2 h glucose levels. ApoA-IV was inversely and THBS1 was positively associated with 2 h glucose levels. MASP1 associations with prediabetes and fasting glucose resisted Bonferroni correction. Type 2 diabetes associations were partly influenced by glucose-lowering medication.
In conclusion, we discovered novel and independent associations of prediabetes and related traits with MASP1, and some evidence for associations with THBS1, GPLD1 and ApoA-IV, suggesting a role for these proteins in the pathophysiology of type 2 diabetes.

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Protein- and Background- based ANOVA inference in proteomic analyses

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Background
Expression change inference in mass spectrometry-based proteomics faces several challenges. Material and acquisition time restrict noticeably the number of measured biological and technical replicates reducing the statistical power of protein ANOVA-based analyses. In order to circumvent this issue, proteins may be compared to a background of non-changing proteins assuming that quantification variability is strongly related to detected signal to noise. Comparing each protein to several proteins (of similar signal) is also advantageous since it allows taking into account technical and biological variability. While this method works reasonably well in most of proteomics profiling experiments, it requires quantification of a substantial number of background proteins. Many experimental approaches like immunoprecipitations or subcellular enrichments would not provide background enough for a correct expression change inference. Additionally, a higher number of replicates make protein ANOVA-based inference over perform inference precision of background-based ANOVA. We are interested on exploring the limitations of each methodology.

Methods
Two samples A and B were prepared by mixing three different proteomes (human Jurkat T-cells, yeast, Danio rerio) in known ratios (A: 55% human, 20% yeast, 25% Danio rerio; B: 55%, 35%, and 10%) reaching a total protein quantity of 1 µg on each sample. Cell cultures were first digested with trypsin, mixed in the above-mentioned ratios, labelled with Thermo TMT-10plex reagents (Sample A: 126-128C, Sample B: 129N-131), and then fractionated by high pH reversed-phase peptide fractionation resulting in 10 fractions for each sample. Each fraction was acquired twice in a Thermo LTQ-Orbitrap Elite instrument.

Results
We analyzed different fraction mixes (modifying the number of quantified proteins) and different number of replicates (by using different numbers of TMT channels) in Proteome Discoverer 2.2.

Conclusions
Data analysis allowed us to find advantages and limitations of both ANOVA strategies to better define which method should be applied.

Keywords
Bioinformatics, statistics

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In vivo SILAC-based Proteomics Identifies a Novel Potential Target in Castration Resistant Prostate Cancer

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Background:
Prostate cancer (PC) is a global major health concern. The cornerstone of treatment for advanced metastatic disease is Androgen Deprivation Therapy (ADT). Despite initial control, all men on ADT eventually develop aggressive castrate resistant prostate cancer (CRPC). Tumour and host interactions play an important role in the development of CRPC. To identify novel therapeutic targets, a quantitative SILAC proteomic analysis was performed on in vivo tumour models of CRPC.

Methods:
Hormone naïve (HN) models (CWR, LNCaP and VCaP) and matching isogenic CRPC models (22RV1, LNCAP AI and VCaPCR, respectively) were generated by in vivo orthotopic implantation of cells in CD-1 nude mice +/- surgical castration. Tumours were analysed in biological triplicates, using a super SILAC standard (heavy labelled CWR, LNCaP, LNCaP AI and VCaP) for quantification and processed by filter aided sample preparation. Samples were fractionated using a high pH C18 column into 21 fractions per sample prior to LC-MS analysis on an LTQ Orbitrap Velos.

Data were analysed using MaxQuant and Perseus platforms. Significantly changing proteins in CRPC were overlapped and analysed for commonly highlighted pathways in CRPC using Reactome. Candidate proteins were validated by immunohistochemistry (IHC) of tumour sections.

Results:
4541 human proteins were identified and quantified in at least two biological replicates. Common pathways were identified in CRPC involving metabolism of lipids and lipoproteins regulated in all three CRPC models. Nuclear protein Human Schlafen 5 (SLFN5) was highlighted to be upregulated in CRPC, >2 fold change in all three HN/CRPC model pairs, as well as validated by IHC.

Conclusions:
Quantitative Proteomic analysis of CRPC identifies lipid metabolic pathway components and a novel nuclear factor SLFN5 as a potential target in CRPC.

Keywords: Prostate, Cancer, SILAC, Tumour, Resistance

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Integrative organelle proteomics for deep characterization of the mitochondrial proteome

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Background

Mitochondria play important roles in numerous cellular processes including energy metabolism and iron-sulfur protein biogenesis. To comprehensively understand mitochondrial functions, detailed knowledge of the mitochondrial proteome is essential. Previous studies targeting the mitochondrial proteome of the eukaryotic model organism Saccharomyces cerevisiae compiled a valuable, first mitochondrial protein inventory. Yet, new mitochondrial proteins are still being identified indicating that current knowledge of the mitochondrial proteome is incomplete. We here present an integrative quantitative proteomic study for deep characterization of the mitochondrial proteome.

Methods

Subcellular and submitochondrial fractions were analyzed by SILAC-MS. A multi-protease digestion and separation approach was used to define mitochondrial proteins by classification. Copy numbers were determined on glucose, galactose and glycerol. Interactomes were established by quantitative AP-MS.

Results

To comprehensively characterize the mitochondrial proteome, we analyzed fractions of different purity and classified more than 3,300 proteins of mitochondria and mitochondria-associated fractions. By absolute quantification, we obtained a deep understanding of the mitochondrial proteome and its dynamics. We extend current knowledge by identifying 82 new mitochondrial proteins including proteins with multiple localizations. The mitochondrial localizations were confirmed by biochemical or fluorescence microscopy analyses. We determined submitochondrial localizations and revealed for selected new mitochondrial proteins their integration in large membrane protein assemblies with central functions in mitochondria.

Conclusions

Our integrative study allowed for the definition of a high confidence mitochondrial proteome consisting of 900 proteins with detailed information about copy numbers, submitochondrial localization and membrane protein topology. Our multi-dimensional data provide firm evidence for 82 novel mitochondrial proteins and further 119 proteins with a previous ambiguous localization to mitochondria. Thus, it represents a rich source for further expanding our knowledge about mitochondrial organization and functions, which is exemplified by the identification of novel links between respiratory chain complexes, AAA proteases and the mitochondrial contact site and cristae organizing system.

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Proteomics space odyssey: Strategy of proteoforms scouting

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Background
An important factor that limits the study of the proteome is its heterogeneity i.e. an abundance of variations of amino acid sequences and post-translational modifications of proteins. Necessity for identification and investigation of alternative splicing events (AS), single amino acid polymorphisms (SAP) and post-translational modifications (PTM) at the protein level is determined by the influence of these processes on the level of expression and functional properties of proteins.

Methods
To study proteome heterogeneity we carried out a comprehensive research of gene expression for HepG2 cell line and liver tissue by means of transcriptomic data analysis and proteomic methods (2DE with separation of gel into spots and following mass spectrometry of each spot).

Results
Transcriptomic analysis of HepG2 cell line and liver tissue resulted in exome-specific proteoform catalogues, containing 52 thousand and 50 thousand amino acid sequences, encoded by 12 thousand 13 and genes, correspondingly. 2DE proteomic profiling with further mass spectrometry analysis allowed to discover over 30 thousand proteoforms encoded by 4 thousand genes for HepG2 cell line, and 15 thousand proteoforms encoded by 3 thousand genes for hepatocytes, correspondingly. Primary analysis of the combined transcriptoproteomic approach revealed its promising outlook, wherein, according to our data, only one in three proteoforms can be visualized by means of gel electrophoresis solo. As exemplified by human chromosome 18 – object of Russian part of Human Proteome Project – we managed to describe 32 and 38 proteoforms encoded by 15 and 25 genes of this chromosome for HepG2 cells and hepatocytes, correspondingly.

Conclusions
Obtained results consist not only of evaluating (the prediction of existence, descriptive characteristics and selective experimental validation) of proteoforms implemented at the protein level, but also of improving experimental approaches to cells and tissues proteotyping.

Keywords
Proteoforms, Transcripto-Proteome, Human, RNA-Seq, 2DE, MS/MS

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Discovery of new proteins and modifications with community-scale proteomics big data

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Translating the growing volumes of proteomics mass spectrometry data into reusable evidence of the occurrence and provenance of proteomics events requires the development of novel community-scale computational workflows. MassIVE (http://massive.ucsd.edu) proposes to address this challenge in three stages.

First, systematic annotation of human proteomics big data requires automated reanalysis of all public data using open source workflows with detailed records of search parameters and of individual Peptide Spectrum Matches (PSMs). As such, our large-scale reanalysis of tens of terabytes of human data has now increased the total number of proper public PSMs by over 10-fold to over 260 million PSMs whose coverage includes over 95% of public human HCD data.

Second, proper synthesis of community-scale search results into a reusable knowledge base (KB) requires scalable workflows imposing strict statistical controls. Our MassIVE-KB spectral library has thus properly assembled 2+ million precursors from over 1.5 million peptides covering over 6.2 million amino acids in the human proteome, all of which at least double the numbers covered by the popular NIST spectral libraries. Moreover, MassIVE-KB detects 723 novel proteins (PE 2-5) for a total of 16,852 proteins observed in non-synthetic LCMS runs and 19,610 total proteins when including the recent ProteomeTools data.

Third, we show how advanced identification algorithms combine with public data to reveal dozens of unexpected putative modifications supported by multiple highly-correlated spectra. These show that protein regions can be observed in over 100 different variants with various combinations of post-translational modifications and cleavage events, thus suggesting that current coverage of proteome diversity (at ~1.3 variants per protein region) is far below what is observable in experimental data.

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Correlation of Histopathologic Characteristics to Protein Expression and Function in Malignant Melanoma

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Background: Metastatic melanoma has the highest increase of incidence of malignancies in the western world, which upon progression has neither a prognostic marker nor a specific and lasting treatment. With the aim to identify better clinical diagnostics biomarkers, as well as predictive and prognostic markers, we focused on relating high resolution proteomics data to careful histopathological evaluation of the tumor samples and patient survival information.

Methods: Regional lymph node metastases obtained from ten patients with metastatic melanoma (stage III) were analyzed by histopathology and protein expression using mass spectrometry. The protein expression data was related to the histopathology in tumor tissue sections adjacent to the area used for mass spectrometry analysis. Clinical follow-up data provided information on disease progression which could be linked to protein expression.
Results: In this feasibility study, several proteins were identified that positively correlated to tumor tissue content including IF6, ARF4, MUC18, UBC12, CSPG4, PCNA, PMEL and MAGD2. MYC, HNF4A and TGFB1 were also identified as top upstream regulators correlating to tumor tissue content. Other proteins were inversely correlated to tumor tissue content, the most significant being; TENX, EHD2, ZA2G, AOC3, FETUA and THRB. A number of proteins were significantly related to clinical outcome, among these, HEXB, PKM and GPNMB stood out, as hallmarks of processes involved in progression of disease and poor survival.

Conclusion: In this study, promising results show the feasibility of relating proteomics to histopathology and clinical outcome. The combined analysis of histological features including the sample cellular composition with protein expression of each metastasis enabled the identification of differentially expressed proteins. Further studies are necessary to determine whether these putative biomarkers can be utilized in diagnostics and prognostic prediction of metastatic melanoma.

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Mutations in HIV GAG Peptides and Their Effect on Cellular Immune Response in Pakistani Patients

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Background:
The infected cells present viral antigens to the CTLs leading to the generation of immune response and destruction of infected cells. Presentation of viral epitops by antigen presenting cell involve many steps. Response of the CTLs with particular HLA type is associated with slower disease progression. Our study is to identify Mutations in gag gene of HIV-1 and their effect on proteosomal degradation of peptides and association of HLA types with selected viral mutations.
Methods:
Blood samples were collected from HIV patients, followed by DNA extraction which was used in HLA typing and in Nested PCR to amplify the HIV gag gene followed by sequencing. The sequences were analyzed using bioinformatics tools including ClustalX to identify mutations and for potential proteosomal degradation sites using Netchop. The selected peptides containing the proteosomal degradation sites were synthesized and used in protoeosomal degradation assay. Digested peptides were run on SDS gel to observe the degradation pattern. The digest products were send for MALDI-MS to confirm the exact cutting sites of the peptides.
Results
We identified T303V mutation in gag region in most of the Pakistani isolates which is a stronger proteosomal degradation sites as compared to T303T in majority of the sequences that we retrieved from Los Alamos HIV sequence database. The HLA types of the patients were relatively diverse but HLA A*68 was found more prevalent than other HLA types, showing some correlation with T303V mutation.
Conclusion
The HIV infection in the region is relatively new and the virus is evolving according to the local environment and immune pressure by the patients. We have identified T303V mutation in locally spread virus. We intend to use the peptide containing this particular residue in ELISpot experiments to further elucidate the interaction between HIV and the host immune system.
Keywords
HIV, Pakistan, Mutation, GAG

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Comprehensive Tissue proteomic analysis of human gliomas

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Background
Gliomas are most common CNS tumors. WHO classified gliomas into four different grades based on microscopic characteristics, immunohistochemistry and mutational status of a few genes like IDH1 and ATRX. Of the four different grades of gliomas, grade-IV gliomas are most aggressive with an overall survival period of 12 months from the time of diagnosis. Objective of our study is to identify grade specific protein markers and to understand the aggressive nature of high-grade gliomas.
Methods
Proteins from the brain tissue samples were extracted using Trizol based protein extraction method. Tissue proteomic analysis of different grades of gliomas and control samples was carried out using gel based (2D-DIGE) and mass spectrometry based (iTRAQ-LC-MS) approaches. Significantly altered proteins were subjected to statistical and pathway analysis using Metaboanalyst and DAVID tools respectively. CRYAB and SNCA gene constructs were transfected into human GBM cell line (LN18) and proliferation rates of these transfected cells were studied using MTT assay.
Results
Present study revealed differential expression of proteins associated with glycolysis, Kreb's cycle, electron transport chain, lactate metabolism, blood coagulation and cell cycle. A few proteins like LDHA, SERPINA1, ANXA5, NPM, FTL and TMSL1 showed increased expression with increase in the grade of the tumors. Proteins like BASP1, SNCA, SYN1, CKMT and COX5A were found to be down-regulated in gliomas. Proliferation assay performed using CRYAB and SNCA gene transfected LN18 cells revealed that SNCA plays a key role in tumor suppression.
Conclusions
These finding will be useful in understanding the glioma pathogenesis better and the differentially expressed proteins, which showed either positive or negative correlation with increase in the malignancy of the tumor could be potential biomarkers.

Keywords
Glioma, Tissue proteomic analysis, iTRAQ, Transfection, Markers

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Quantitative analysis of signaling pathways using 11plex TMT reagents and comprehensive phosphopeptide enrichment strategies

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Background
There is broad interest in quantifying protein phosphorylation alterations in cellular signaling pathways under different conditions. The transient nature and low stoichiometry of phosphorylation, and low abundance of many targets makes this challenging. Enrichment is necessary for better detection of the low abundant phosphorylated proteins and multiplexed quantitation reagents parallelize processing while permitting several experimental conditions. We have combined SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) method with 11 plex Tandem Mass Tag (TMT) isobaric labeling reagents to evaluate changes in phosphorylated proteins expressions under different stimulation conditions.

Methods
HeLa cells were grown with 10 different conditions of starvation/stimulations (Nocodazole/TPA/hIGF-1/hEFG/hPDGF-bb/FBS/charcoal stripped FBS) before being subjected to in-solution digestion. Thermo Scientific TMT10plex tags plus a novel TMT11-131C reagent were used to label 0.45mg of HeLa digest for each condition. 5mg of combined TMT11plex labeled peptides was subjected to Thermo Scientific Pierce Hi-SelectTM TiO2 phosphopeptide enrichment kit (PN#A32993). TiO2 flow-through/wash fractions were enriched with the Thermo Scientific Pierce Hi-SelectTM Fe-NTA phophopeptide enrichment kit (PN#A32992). Both eluents were combined and fractionated using the Thermo Scientific Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (PN#84868) before LC-MS analysis using Thermo Scientific Orbitrap Fusion instrument. Thermo Scientific Proteome Discoverer 2.1 software was used to localize the phosphorylation sites.

Results
TMT11plex with the SMOAC method allowed comprehensive identification and quantitation of phosphopeptides across different conditions. A peptide assay before TMT labeling and after phosphopeptide fractionation allowed for normalization of peptide amounts from different condition. High pH reversed-phase fractionation after phosphopeptide enrichment resulted in better throughput and proteome depth for profiling changes in phosphopeptide expressions. Excellent selectivity and specificity for phosphopeptides were achieved with this improved SMOAC workflow.

Conclusions
This comprehensive phosphopeptide analysis allowed quantitation of phosphorylation changes for thousands of signaling pathways proteins under different conditions.

Keywords
Phosphopeptides enrichment, Signaling pathways, Isobaric tags multiplexing, Phosphorylation

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PRDX2- redox-oligomeric state in Obstructive Sleep Apnea patients with diabetes mellitus

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Background: Obstructive sleep apnea (OSA) is a common public health concern causing deleterious cardiometabolic dysfunction. We previously showed that OSA induces alterations in red blood cell (RBC) proteome. Alterations in the redox/oligomeric states of PRDX2 correlated with severity and metabolic status of OSA and six month of positive-airway-pressure (PAP) treatment increased PRDX2 redox/oligomeric state associated with chaperone protective function (Feliciano et al 2017). Herein, we aimed to investigate this protein and its relationship with PAP response in OSA patients with diabetes mellitus to better understand the basic mechanisms associated with OSA and OSA outcomes.
Methods: RBC samples from control snorers (n=22 being 3 diabetics) and OSA patients before and after six month of PAP-treatment (n=29 being 8 diabetics) were analysed by non-reducing western blot using antibody against PRDX2 or PRDXSO2 to measure the total and overoxidized levels of monomeric/dimeric/multimeric forms of PRDX2. Groups were statistically compared and correlated with clinical/biochemical data and significance set up at 5% (p value < 0.05).
Results: Monomeric forms of PRDX2 were higher overoxidized in OSA non-diabetic RBCs that decreased after PAP treatment followed by an increase of multimeric-overoxidized forms associated with chaperone protective function. In OSA diabetic RBCs, the level of PRDX2 monomers although higher abundant its overoxidation level was much lower and did not significant change after treatment. Moreover, the level of PAP-induced PRDX2-overoxidized-multimers was also lower in these patients. In diabetic patients, the steady-state level of PRDX2 monomers and dissulfidic-dimers associated with peroxidatic function positively correlated with OSA severity and glycemic status, respectively.
Conclusions: The redox/oligomeric state of RBC PRDX2 were differentially modulated in OSA diabetic patients compared to OSA without this comorbidity. PAP-induced PRDX2 chaperone protective function showed decreased in OSA patients with diabetes. The clinical impact of these findings needs further investigation.
Keywords: OSA, PRDX2, diabetes, positive-airway-pressure (PAP)

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Human neocortex proteome profiling during progression of tau pathology in Alzheimer’s disease

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Background: In this work we evaluate alterations that occur in the human neocortex proteome during progression of tau pathology in Alzheimer´s disease (AD), using a mass spectrometry based approach.

Methods: A total of 57 post mortem human brain samples were obtained from the Human Brain Tissue Bank (Semmelweis University) and classified into disease stages according to tau pathology (Braak/Braak I, II, III, IV, V, VI and controls). Frozen tissue samples were prepared with a modified filter-aided sample preparation method. The nLC-MS/MS analysis was performed on a Q Exactive mass spectrometer equipped with an EASY- nLC 1000 system. Database search was done with Proteome Discoverer v2.1.

Results: A total of 3761 and 3633 protein groups were identified in frontal and temporal cortex, respectively (high protein FDR and at least 2 peptides/protein). The distribution of protein groups among the Braak/Braak stages was observed and few proteins were identified in specific disease groups. 438 and 460 proteins were differentially expressed between the disease stages in frontal and temporal cortex, respectively (Student T test, $p < 0.05$, Fold Change≥ 2). Some proteins or functionally related proteins show a conserved expression pattern during disease progression in both brain regions. Remarkably, ribosomal proteins have similar patterns in frontal and temporal cortex.

Conclusions: The proteomic profiling of these two neocortical regions showed commonly regulated proteins in AD and will possibly give more insights into disease progression mechanism.

Keywords: Alzheimer´s disease, tau pathology, brain proteome

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Application of CE-MS for the Quantification of Mono-phosphorylated Isobaric Peptides

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It is known that CZE is able to separate peptides with identical amino acid composition but with different sequence provided the sequence variation causes a variation in pKa values of the corresponding peptides. This can be induced, e.g. by local differences in hydrophobicity, conformation, extent of hydration and even more importantly by interaction between charged moieties. For this reason, variation of buffer pH and concentration and the addition of organic solvents, e.g. TFE can be used to optimize resolution. Moreover, the number and position of adjacent charges present either at the amino terminus or in the interior of the peptide may selectively alter the overall charge of those peptides.

In this study, CE-MS was used as complementary approach for the quantification of mono-phosphorylated isobaric peptides, still a very challenging task in the proteomics field. If such positional isomers cannot be separated, an unambiguous assignment of site-specific phosphorylation effects is problematic, a quantification often impossible. In the course of a kinase activity study isobaric mono-phosphorylated peptides were identified as potential substrates and therefore, the CE-MS approach was evaluated for their identification and quantification. The complex peptide sample was pre-fractionated by RP-HPLC and the fractions of interest were analyzed by CE-MS using a neutral capillary. Among other interesting candidates as potential kinase substrates, six phosphopeptide isomers were identified that differ in their migration time by at least 0.4 min. The isobaric phosphopeptides were clearly separated from each other and in every case the isomer with a phosphate group bound to serine or threonine residues that were in vicinity to a basic amino acid or the N-terminus exhibited a lower electrophoretic mobility.

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Proteomics of rat hippocampus reveals proteins related to operant learning

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Operant conditioning is a form of associative learning in which the subject learns to anticipate future events coming as consequence of its behavior. This learning process occurs in humans and other animals. Several neurological and psychiatric disease impair learning, disrupt memory and affect behaviors. Rats have been used in behavior and learning research for the generation of molecular information related to such processes. Proteomics can be very useful for the understanding of biochemical mechanisms related to behavior, memory and learning. However, little proteomic data related to operant learning is available. The present work aimed at using LC-MS/MS (Orbitrap Elite) to identify proteins with differential abundance in subjects trained under the operant paradigm. Rats were submitted to discriminative operant conditioning in Skinner boxes, followed by analyses of their hippocampal proteomes. A total of 6,082 proteins were identified, from which 39 were differentially abundant between trained and control rats under stringent statistical analyses. Examples of proteins down-regulated are beta-soluble NSF attachment protein, myristoylated alanine-rich C-kinase substrate, protein Inf2, SPARC-like 1 isoform CRA_a and actin-related protein 2/3 complex subunit 4, while up-regulated examples are calreticulin, cytoplasmic dynein 1 heavy chain 1, gelsolin, eukaryotic translation initiation factor 4 gamma 3 and methyltransferase like 7A. Such proteins are involved in regulation of cytoskeleton organization, vesicle traffic, signal transduction and gene expression control.

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Which proteins are responsible for the selective neuronal vulnerability in Parkinson's disease?

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Within neurodegenerative disorders specific neuron populations are degenerating, e.g. dopaminergic neurons in the substantia nigra during Parkinson's disease. Furthermore, not all dopaminergic neurons within the substantia nigra are equally affected. Studies revealed that ventral located neurons within the substantia nigra have a higher prevalence to degenerate during Parkinson's disease compared to neurons in the dorsal tier of the substantia nigra. Reasons for this selective neuronal vulnerability are still an open issue. To gain a better understanding of molecular differences between these types of neurons, these neurons were isolated specifically with laser microdissection [1]. While methods in the past had the disadvantage that it was not possible to isolate specific cell populations of a complex tissue, laser microdissection overcomes this problem. Using this approach in combination with data independent acquisition mass spectrometry dorsal and ventral located neuron populations within the substantia nigra of cognitively normal individuals were analyzed. The results of this test study revealed first hints in differences between these two types of neurons. The study is now extended to get a higher statistical significance. With that we expect to get a deeper understanding of the neuroprotective mechanisms.

Molina M, Steinbach S, Park YM, Yun SY, Di Lorenzo Alho AT, Heinsen H, Grinberg LT, Marcus K, Leite RE, May C: Enrichment of single neurons and defined brain regions from human brain tissue samples for subsequent proteome analysis. J Neural Transm 2015, 122(7):993-1005.

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PSI standards compatible binary mass spectrometry data format for efficient read/write speed and storage requirements

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Background
Raw mass spectrometry (MS) datasets contain two types of data: (a) numeric data e. g. m/z, intensities; (b) metadata related to instrument and experimental settings.

The Proteomics Standards Initiative (PSI) have developed the open mzML community standard for sharing raw MS data. One of the bottlenecks of mzML is that it is a text based XML file format and so all numeric MS data are converted into text strings using Base64 encoding. This inflates the size of output files 4- to 18-fold in comparison to files in the original propriety vendor format. To address these issues the lossy compression scheme Numpress was recently introduced. However, it does not compress the text metadata.

Like mz5, we propose to use the HDF5 file format. mz5 is a complex mapping of the mzML tags to HDF5, we propose a simple hybrid format whereby the numeric data is accelerated by HDF5 while the metadata is kept in fully PSI-standard mzML.

Methods
All Base64 encoded data from mzML are stored natively as HDF5 datasets. Utilising standard HDF5 functionality, we implemented a very simple lossy compression method. Additionally, HDF5 provides mechanisms for partial reading or writing.

Results
We compared our new mzMLb file format to vendor raw file, mzML, mz5, and Numpress within both mzML and mzMLb. When using lossy compression, the maximum relative error of the data stored in mzMLb was ensured to be equal to or less than that of NumPress output. In all cases resulting files were significantly smaller than mzML and a similar size to the vendor raw file.

Conclusions
We have implemented the mzMLb format in the ProteoWizard package, and demonstrate simple readers in R and Julia. Implementation in other software tools should be straightforward as HDF5 is supported in all common programming languages.

Keywords: Proteomics Standards Initiative, mzML, HDF5

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AIRWAY PROTEOME SIGNATURE ASSOCIATED WITH SECOND-HAND SMOKE EXPOSURE

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Background: Non-smokers exposed to second-hand smoke (SHS) are at risk to develop tobacco smoke associated diseases and improved risk prediction and understanding of SHS-induced pathogenesis are needed. We have previously shown that non-smokers workers of a group of Lisbon smoking restaurants presented higher levels of urinary cotinine and changes in their plasma proteome that might be associated with SHS exposure (1). Herein, to better investigate the molecular effects of SHS we performed a proteomic study at the respiratory level of those occupationally exposed subjects.
Methods: Nasal brushing samples (n=51) were selected from those worker's biobank for proteomics study. All samples were from healthy subjects with normal spirometry values (FVE1/FVC <70%) for pulmonary diseases. Subjects were classified as: Never-smokers/non-exposed (N; n=10), Never-smokers/Exposed (NE; n=11), Former-smokers/non-exposed (F; n=8), Former-smokers/Exposed (FE; n=10); Smokers/non-exposed (S; n=8) and Smoker/Exposed (SE; n=4) to SHS. All non-smokers exposed presented higher levels of urinary cotinine compared to non-exposed ones. Samples were analysed and compared by ESI-LTQ-Orbitrap XL mass spectrometer and data by PatternLab for Proteomics 4.0 and DAVID bioinformatics resources 6.8.
Results: About 59 and 76 proteins were identified differently/exclusively abundant in NE and FE exposed groups, respectively, compared to non-exposed ones. The proteomic differences observed between NE/FE and smokers were much lesser than those between N/F and smokers. The differentially proteins are mainly associated with glycolysis/gluconeogenesis, metabolism of drug/xenobiotics by cytochrome P450, regulation of actin/tubulin-cytoskeleton, retinol metabolism pathways, among others. Aging/telomere length, COPD/COPD emphysema, anoxia, bronchitis, esophageal/head/neck/laryngeal neoplasm diseases were found associated with those proteins.
Conclusions: Our findings strongly support that prolonged occupational exposure to SHS can lead to subclinical pathologic molecular events associated with tobacco smoking. Further validation studies may contribute to the better understanding of pathogenesis mechanisms due to SHS exposure and prevention of tobacco-induced diseases.
Key words- second-hand smoke, occupational health, biosignature, biomarkers, airway

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Temporal quantitative phosphoproteomics of ADP stimulation reveals novel central nodes in platelet activation and inhibition

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Adenosine diphosphate enhances platelet activation by virtually any other stimulant to complete aggregation. It binds specifically to the G-protein–coupled membrane receptors P2Y1 and P2Y12 [1], stimulating intracellular signaling cascades. P2Y12 inhibitors are among the most successful antiplatelet drugs, however, show remarkable variability in efficacy. We applied quantitative temporal phosphoproteomics to study ADP-mediated signaling at unprecedented molecular resolution. We provide temporal profiles of 4797 phosphopeptides, 608 of which showed significant regulation. Our data demonstrate that ADP-triggered phosphorylation occurs predominantly within the first 10 seconds, with many short rather than sustained changes. This study demonstrates an extensive spectrum of human platelet protein phosphorylation in response to ADP and Iloprost, which inversely overlap and represent major activating and inhibitory pathways.

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EGFR Interactome Reveals Multiple Pathways and Regulatory Mechanism of Drug-resistance in Non-Small Cell Lung Cancer

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Background
Dysregulation of EGFR signaling leads to aberrant cell survival, growth and metastasis in cancer cells. Tyrosine kinase inhibitors targeting mutant-EGFR (mtEGFR), have effectively enhanced survival rate of advanced NSCLC patients, but most of them eventually develop drug resistance. Although several pathways responsible for TKI-resistance have been reported, the mechanisms on the signaling pathways how mtEGFR drive TKI-resistance remain to be elucidated. EGFR regulates cellular function through diverse and complex interactions. It's intriguing to study whether the mtEGFR may change the recruitment partners and affect downstream signaling.
Methods
Two pairs of NSCLC cell lines with primary/secondary EGFR mutation, H3255(L858R)/H1975(L858R-T790M) and PC9(Del19)/CL68(Del19-T790M), were used as drug sensitive/resistant NSCLC models. Affinity-purification mass-spectrometry (AP-MS) was used to analyze protein-protein interactions (PPIs) of different mtEGFR. Further functional validations of the identified potential interactors will be validated by western-blot, immunofluorescence and PCR.
Results
We identified a total of 640 and 760 interaction proteins from H3255/H1975 and PC9/CL68 interactomes, respectively. After using quality control and interaction scoring to eliminate non-specific binding for highly confident EGFR PPIs, around 100 proteins were obtained to represent potential EGFR-binding partners. Some interactors were commonly present in both interactomes, including ERBB2, JAK1, and MET well-known for EGFR regulated proliferation, oncogenesis and drug-resistance. Bioinformatics analysis further revealed pathways potentially crucial for drug-resistance caused by T790M mutation. For example, proteins involved in endocytosis responsible for degradation, ATP-binding, and vesicle formation had higher intensity in H1975. Moreover, multiprotein-complex units of proteasome were up-regulated in CL68. These results suggest that EGFR internalization/degradation may play a role in drug-resistant mechanisms.
Conclusions
Collectively, we anticipate that our EGFR-interactomes would demonstrate a systematic approach to identify protein/pathway associated with EGFR-dependent TKI-resistance. We will clarify the functional role of potential EGFR interactors in TKI-resistant mechanism in the near future..
Keywords
Non-small cell lung cancer, TKI-resistance, AP-MS, EGFR mutation, Interactome

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Capillary Electrophoresis – Mass Spectrometry for Intact Mass Analysis of Antibodies and Antibody-Drug-Conjugates

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Monoclonal antibodies (mAbs) and antibody-drug-conjugates (ADCs) are a widely used class of therapeutic modalites for a wide variety of diseases and therefore play an increasing role in Biopharma research and development. mAbs and ADCs are very complex chemical compounds with a molecular weight of ~150 kDa. Since regulatory demands require complete characterization, effective and meaningful analytical methods need to be developed. Coupling the separation selectivity of capillary electrophoresis (CE) with high resolution accurate mass spectrometry (HRAM) is a powerful technique, here explored as chip-based CE coupled to Orbitrap MS for mAb and ADC analysis.
The reference NIST mAb has three charge variants with 0, 1 or 2 lysine residues, which can be separated by CE with the ZipChip microfabricated glass chip within 3 minutes, using electric field strengths of up to 1000 V/cm and resulting in peaks of only 3 to 5 seconds wide. Despite the acid separation conditions, the MS of the mAb has a charge envelope ranging from +27 to +32, similar to what would be expected under native conditions Deconvolutuion using Biopharma Finder 2.0 software, provided accurate intact mass for at least 15 glyco forms. It was crucial to have the extended mass range in the 3000 – 8000 Da range of the Q Exactive HF with Biopharma option to record these low charge states of the sample with high transmission. The two glycol chains of the NIST mAb were modified using click chemistry to produce a non-toxic linker/payload. The resulting model ADC was successfully separated and annotated with the correct MW by CEMS into the corresponding charge variants. In summary, CEMS allows fast separation of mAb and ADC charge variants and their accurate MS1 mass determination and will therefore enable novel QC methods for these complex drugs in the biopharma industry.

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Dynamics of the Neuronal Surface Proteotype

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Background
The brain is a complex mixture of neurons and glial cells organised into networks. Information processing of neural networks requires cell-to-cell communication via synapses. Precision in synaptic connections and their structural features in turn are key to establish function in neural circuits. Thus, neurons utilize a plethora of proteoforms to diversify the synaptic microenvironment enabling diversity and plasticity in synaptic connections. Thereby, quantity and nanoscale organization of cell surface proteins determines functional properties of synapses and neurons. Furthermore, precise wiring of neural circuitry is thought to be mediated by molecular diversification of surface molecules and their extracellular interactions. However, global analysis of the proteotype on the neuronal surface has proven challenging, mainly due to lack of technologies to determine protein quantities and their sub-cellular localisation.

Methods
Cell-Surface-Capture (CSC) enables identification and quantification of the glycoprotein population residing in the plasma membrane (surfaceome) on live cells. We advanced CSC using a liquid handling robot leading to >100-fold increased sensitivity enabling application with fewer cell numbers and increased throughput.

Results
Using the automated workflow, we quantified dynamics during neuronal differentiation, synapse formation and synaptic plasticity in primary neuronal cultures. Taking time resolved snapshots of the surface during three weeks of neuronal differentiation we provide high resolution (two days) quantitative profiles for >1100 surface proteins throughout different developmental stages from neurite outgrowth to synapse formation. Furthermore, we quantified the surfaceome during chemical long-term potentiation and identified a set of extracellular proteins that rapidly localise to the surface upon stimulation. Complementary, we quantified neuronal secretome dynamics during differentiation and selected identified ligands to decipher their interaction partners as first step towards the extracellular interactome.

Conclusions
We target the quantitative protein organisation on the surface of neuronal cultures using chemoproteomic technologies, a key step towards deciphering neuronal differentiation from a system-wide perspective.

Keywords
Neuroproteomics

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Comparative proteome analysis of long-term survivor and non-survivor patients with acute myeloid leukemia

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Background:
Acute myeloid leukemia (AML) is a heterogeneous and aggressive blood cancer, characterized by accumulation of limited differentiated blast cells of the myeloid lineage. AML patients usually receive induction chemotherapy as the initial treatment, and although many patients achieve complete remission is AML often associated with leukemia relapse and chemoresistance. There is need for improved risk stratification of these patients, especially regarding who would benefit from allogeneic hematopoietic stem cell transplantation.

Methods:
For proteomic comparison of AML cells from long-term survivors and non-survivors, we used the super-SILAC approach together with FASP-based protein digestion and mixed-mode (SDB-RPS) fractionation to analyze the proteomes of 50 AML patients and 8 controls on a Q Exactive HF mass spectrometer. The patient cohort was selected based on the clinical outcome after treatment with conventional chemotherapy. CD34+ cells isolated from the bone marrow of eight healthy individuals were included as a control group.

Results:
We quantified 5,619 protein groups in at least twenty percent of the patients in each group (i.e. survivors and non-survivors) of which 330 protein groups had significantly different protein abundances in the survivors compared to the non-survivors. Functional analysis revealed that proteins involved in biological processes including mitochondrial translation and proline metabolism had higher abundance in the non-survivors. Conversely, proteins involved in processes involving the immune system and pyridine-containing compound metabolism had lower abundance in the non-survivors. We further extracted 61 proteins which also were significantly different between the patient groups in a label-free experiment.

Conclusions:
Comparative analysis of AML proteomes from patients with different survival success indicate that reprogramming of metabolic processes and the immune system have importance in AML and therapeutic resistance; hence the differentially abundant proteins may represent therapeutic targets, in addition to guide the treatment regimen.

Keywords:
Acute myeloid leukemia, Clinical proteomics, Super-SILAC

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Controlling false discovery rates on large-scale proteome datasets in jPOST

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Background
To develop very large proteomic database such as human proteome database, it is necessary to control the false discovery rate (FDR) on each dataset accumulated in public proteomic repository such as jPOST (Japan ProteOme STandard Repository/Database) repository as a member of ProteomeXchange Consortium. The target-decoy approach (TDA) has been widely used to estimate the FDR, although it has some demerits in applying to very large proteomic datasets. Several scalable approaches to analyze millions of PSMs have been reported. However, it is still challenging to control the FDRs on large-scale datasets from multiple projects and institutions.

Methods
MS raw datasets acquired by Thermo Q-Exactive, Orbitrap Velos and Sciex TripleTOF 5600 for the global and phosphoproteome analysis of human cells were obtained from jPOST. The protein identification was performed by X!Tandem, Mascot, Comet and MaxQuant against UniProt 2016_3.

Results
We applied a posterior scoring approach based on the concept of peptide sequence tags (PSTs) to the PSMs from multiple search engines. To develop a scalable method to control the FDRs, we used the local FDR approach to evaluate the relationship between the PST-based scores and the TDA-based FDRs to calculate the false positive rates (FPR), defined as the probability that a spectrum matches a random peptide with a score over a threshold. The obtained results suggested that the FDRs can be estimated by FPRs through the PST-based scores. We also compared the PST-based scores with the probability-based scores such as Mascot peptide score and posterior error probabilities such as Percolator q-values, and found that our approach tends to provide lower FDRs or more PSM hits at 1% FDR. Because this approach is based on the FPR for each PSM, it would be suitable for large-scale databases based on re-analyzed datasets accumulated in jPOST.

Keywords
jPOST, database, FDR, PST

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Improved top/middle-down antibody characterization using multiple ion activations on an Orbitrap Tribrid mass spectrometer

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Background
Top-down and middle-down mass spectrometry offer advantages for the characterization of biotherapeutics with complete molecular specificity. These technologies allow for reduced sample manipulation, which is potentially crucial when evaluating the quality of production/storage processes, or emerging biosimilars. Reaching the sequence coverage required to confidently verify the antibody primary sequence or map post-translational modifications can be challenging. Here, we apply advanced ion activation techniques, including 213 nm ultraviolet photodissociation (213 nm UVPD) and high capacity electron transfer dissociation (ETD HD), to improve the characterization of antibodies in a sample-efficient fashion.
Methods
MS/MS spectra were collected on 150 kDa intact Rituximab (IgG1), or on large fragments (25-50 kDa), produced by highly-selective proteases (IdeS and GingisKHAN, Genovis). Data was acquired by LC-MS/MS using a modified Orbitrap Fusion Lumos tribrid under three different modes of fragmentation: ETD, EThcD and 213 nm UVPD. UVPD and EThcD data searches were based on 9 and 4 types of product ions, respectively. Fragmentation maps were visualized using ProSight Lite.
Results
The LC-MS/MS results obtained combining ETD and UVPD on intact Rituximab break the previous record of ~32% sequence coverage, reaching a total coverage >40%. For the middle-down experiment using ETD, the sequence coverage for ~ 25 kDa Fd, Lc and Fc/2 subunits, was 47%, 50% and 60%, respectively; UVPD provided 40%, 51% and 71% sequence coverage; EThcD provided 39%, 48% and 54%. When combining the results from ETD, EThcD and UVPD, the sequence coverage reached 72%, 80% and 88%, for Fd, Lc and Fc/2 respectively, using only 6 LC-MS/MS runs.
Conclusions
A high degree of complementarity between UVPD and ETD ion activation methods was observed. As a result, the combination of UVPD with other available ion activations allowed to sequence region of antibody otherwise not fully characterized.
Keywords
UVPD, ETD, EThcD, IgG, antibody, top-down, middle-down

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Multiplexed quantitative analysis using NeuCode SILAC metabolic labeling of signaling protein targets

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Background
NeuCode is a quantitative proteomics technology that enables higher SILAC multiplexing through the use of near isobaric amino acid isotopologues. NeuCode amino acids have the same nominal mass and structure but are labeled with different combinations of 2H, 13C and 15N stable isotopes that can be resolved by modern high-resolution MS instrumentation. In this study, we used NeuCode lysine isotopologues to label cells before enrichment of signaling proteins using multiplexed immunoprecipitation to MS (mIP-MS) analysis. This approach enables multiplexed quantitation of 2 to 7 samples in a single experiment, providing information on both changes in relative target proteins and interacting partners.
Methods
A549 cells were cultured with SILAC media containing 10% dialyzed FBS and one of 8 different lysine isotopologues K000, K202, K040, K602, K341, K080, K642, and K390 (lysine designated number of 13C, 2H, and 15N, respectively). After full incorporation of stable isotopes, cells were serum starved and stimulated with hIGF-1/EGF to stimulate signaling pathways. mIP was performed for simultaneous enrichment of target proteins using IP-MS verified antibodies. Equal amounts of labeled peptides from different conditions were mixed and analyzed with high-resolution Orbitrap instruments using a full scan MS1 at 500K resolving power (@ m/z 200) for quantitation and a lower resolution (60K) MS1 with HCD MS2 for peptide identification. A modified version of MaxQuant software was used to automatically extract the quantitative signatures from the data.
Results
We combined NeuCode SILAC labeling with mIP-MS for simultaneous quantitation of target proteins and to determine specific interacting proteins in multiple sample conditions. Analysis of our samples revealed high correlation to expected ratios and demonstrated feasibility for combining higher multiplex NeuCode analysis with mIP-MS.
Conclusions
NeuCode SILAC labeling and mIP-MS method allowed quantitation of multiple signaling proteins and interactors under stimulated conditions.
Keywords
Signaling pathway quantitation, NeuCode, SILAC multiplexing, mIP-MS

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Characterizing flame retardant-induced neurotoxicity in an hESC neural differentiation model using SWATH® Acquisition and RNA-Seq

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Background:
Due to their persistence in the environment and ability to cause neurodevelopmental toxicity, brominated flame retardants (FRs) are being phased out of the marketplace and the use of alternative FRs is expanding. Consequently, specific alternative FRs including organophosphorous and bromobenzene species are found at appreciable levels in maternal and fetal tissues, with limited understanding regarding the neurodevelopmental health risks associated with exposures in utero. Human embryonic stem cell (hESC) neural differentiation models have tremendous potential for evaluating environmental chemicals in their ability to induce neurodevelopmental toxicity. In this study we compared the concentration-dependent effects of brominated or alternative FRs on cytotoxicity and global protein and RNA levels in hESC-derived neural precursor cells (NPCs).

Methods:
We characterized FR-effects on cytotoxicity (12 unique FRs) and evaluated the impact of subcytotoxic exposures of four FRs at two concentrations on global protein and RNA levels using SWATH MS and RNA-Seq.

Results:
We identified differences in sensitivities across the 12 FR compounds in terms of concentration-dependent effects on NPC viability and death. Specific alternative and classic brominated FRs exhibited similar potencies. Concentration-dependent alterations on both the protein and RNA levels for all four FR compounds were observed. For example, isopropylphenyl phosphate, an organophosphorous alternative FR, induced significant alterations with 3µM (140 genes, 77 proteins) and 10µM (562 genes, 139 proteins) exposures, including important molecules involved in neurogenesis and cell migration. Furthermore, we identified common targets across FRs, suggesting shared modes-of-toxicity.

Conclusions:
This study represents the first investigation to cross-evaluate the hazards associated with FRs simultaneously on transcript and protein levels. Current studies aim to validate and link these FR- molecular targets to functional outcomes in hESC-neuronal development. We expect that these results will provide novel information regarding FR-chemical sensitivity in vitro and mechanisms underlying human neurodevelopmental toxicity.

Keywords: RNA-Seq; SWATH; hESC; neurodevelopment; toxicology

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Pharmacoproteomics of non-human primate cerebrospinal fluid upon BACE inhibition, a drug target for Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia, affecting worldwide nearly 44 million people. In the pathology, the protease β-Site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1) catalyzes the first step in the generation of the amyloid β (Aβ) peptide which is forming oligomers and plaques in the brain. Therefore, many pharmaceutical companies develop BACE inhibitors that are currently in clinical trials. However, in drug development, it is essential to monitor the efficiency of a drug but also possible off-target or unwanted mechanism based side-effects. Indeed, BACE1 has more than 40 potential substrates whose biological functions might be altered by reduced cleavage. Here, we identified global on-target and off-target effects of a BACE inhibitor using pharmacoproteomics of non-human primate cerebrospinal fluid (CSF). The CSF of monkeys was collected before and after the treatment to allow for individual baseline normalization. More than 600 proteins were relatively quantified by label free quantification. The CSF levels of several known BACE1 substrate cleavage products, e.g. SEZ6 and SEZ6L, were found to be reduced. Additionally, we were able to identify new BACE substrate candidates such as VCAM1 and FGFR1. Seizure protein 6 (SEZ6) showed the strongest reduction which was similar to shed APPβ. Since SEZ6 is a protein which is almost exclusively expressed in the brain, it may be used as a companion diagnostic for BACE1 inhibition in vivo. In summary, this study shows that CSF pharmacoproteomics is a valuable tool which should be incorporated into preclinical trials in neuroscience drug development as an unbiased approach.

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Metabolites Imaging and Shotgun proteomics to decipher the role of epididymis in sperm maturation

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Background: Infertility or subfertility is a widespread problem with approximately 25% of couples seeking medical help. For about half the cases it lies in the male partner. Male infertility is caused by problems that affect sperm production, maturation and/or transport. In this study the role played by the epididymis in the post-testicular maturation of spermatozoa during its transit through this organ was investigated using an innovative combination of FT-ICR imaging mass spectrometry of metabolites and shotgun proteomics.

Methods: Distribution of metabolites in the 3 regions of rat epididymis (caput, corpus and cauda) was monitored by high-resolution FT-ICR MALDI imaging. Protein expression along the epididymal tube was monitored by quantitative label-free proteomics. Correlation between metabolite and protein expression was performed using dedicated tools developed by the European consortium METASPACE.

Results: Large-scale metabolites and protein datasets were mapped in the 3 regions of the epididymis using KEGG Pathway. Molecular maps specific to each region of the organ could be drawn and are further explored to investigate the function of specific epididymal proteins in the maturation of the transiting spermatozoa. As a priority, focus is given on the study of oxidoreductases, calcium ion binding proteins, transport regulation proteins, and of proteins involved in glutathione metabolism, in Vasopressin-regulated water reabsorption or in protein processing in endoplasmic reticulum. These pathways are key molecular features of the epididymis biology.

Conclusion: To our knowledge this is the first time FT-ICR Imaging mass spectrometry of metabolites is coupled to quantitative shotgun proteomics to model in situ molecular maps and highlight metabolic pathways deserving further in-depth studies to understand the physiology and pathophysiology of an organ.

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Proteomics evidence for commonality in altered energy and apoptotic pathways in Alzheimer's Disease and Schizophrenia

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Background:
The energy requirements of the brain are very extraordinary and tightly regulated to ensure adequate spatial and temporal delivery of energy substrates in line with neuronal activity. Alteration in energy requirement is a crucial determinant of brain functioning and may lead to several disorders. Here we report that a common theme emerges through functional interactomics which connects the energy disturbances in case of schizophrenia and AD through proteomics evidence.
Methods:
Proteome analysis of SZ & AD regions (hippocampus, substantia nigra and cortex) was performed using Nano-LC MS /ESI -LC MS/MS, 2-DE & WB . The extent of protein expression variation was determined in contrast to age-matched controls and analyzed among the regions using KEGG and IPA analysis.
Results:
Link in metabolic and apoptotic pathways altered in the different brain disorders may provide some insight in the predisposition of AD and SZ patients towards similar metabolic diseases.
Our data revealed differential expression of 16 potent metabolic proteins found to be involved in energy metabolism in brain regions of schizophrenics in common with that of AD. An interesting aspect of this study is the altered phosphorylation of TPI and GAPDH and its association with neurodegeneration where it induces protein misfolding resulting in formation of protein aggregates observed both in AD and SZ.
Conclusion:
We suggest that TPI has a tremendous ability to enhance the protein aggregation process and hence its accumulation in brain areas cause defective energy metabolism and impaired ATP production.
IPA identifies two common hubs of AD and SZ where the metabolic proteins converge is TP53 and APP . This provide evidence that metabolic dysfunction and energy deficit leading to apoptosis is common pathophysiology of Schizophrenia(SZ) and Alzheimer's disease (AD).
The authors acknowledges the support of Dr AR Asif,Germany for MS analysis
Key words:
AD,SZ, Proteomics,Energy Metabolism, Apoptosis, MS.

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Protein array enabled profiling of autoantibody repertoires in ALS, ALS-FTD and FTD

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The general understanding of the global reactivity patterns in the human autoantibody repertoires are still at an early phase. More and more diseases and conditions are speculated to have autoimmune components but very few novel targets are clearly associated to disease conditions. In order to explore autoantidbody reactivities and to screen for novel autoantigens, we have for several years developed, produced and utilized various formats of protein fragment arrays with antigens from the Human Protein Atlas.

Through a combination of various planar and bead-based microarray formats, including an array with 42.000 protein fragments representing 19.055 unique proteins, assays are set up both for broad screening studies as well as targeted analysis for verification and validation of initial findings. The latter format utilize a format where 384 samples can be analyzed in parallel on 384 antigens. We see in general a very large degree of heterogeneity between individuals and also often relatively high numbers of antigens targeted by each individuals repertoires of IgGs, which is also the case for healthy individuals.

Within a larger effort of autoantibody profiling in large number of CSF and plasma samples in a broad neurodegenerative and psychiatric disorder context, have we here focused on a comparison between the autoantibody repertoires in CSF from 80 patients with FTD (frontotemporal dementia) and 320 patients with ALS (amyotrophic lateral sclerosis) and as well as 20 patients with both diagnosis, ie ALS-FTD.

There is in general very little done on any type of broad screening for novel autoantibody targets within these two associated but still very differents diseases. The massive screening done here on 42.000 protein fragments has provided many interesting potential autoantigens that possible can be further associated to ALS and FTD.

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Prioritized iRT in spectral libraries generated from multiple sources for the targeted analysis of DIA

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Targeted analysis of DIA allows reproducible and precise proteome profiling with deep proteome coverage. For highest possible coverage and reproducibility, a high quality spectral library with high precision iRT is of advantage (Bruderer et al. 2016). Spectral libraries with high precision iRTs can be generated from either DDA or DIA data or a combination thereof.

1’261 DDA runs (Kim et al. 2014) were downloaded and searched with the Pulsar search engine (Biognosys) against UniProt (20’203 entries). Six human samples (healthy and cancerous tissue, Biognosys iRT peptides spiked in) were acquired using single shot DIA on an EASY-nLC 1200 / Orbitrap Fusion Lumos (Thermo Fisher Scientific) with 4h gradients. The six DIA runs were searched against UniProt (20’203 entries) using Pulsar’s direct DIA functionality. A combined spectral library was generated controlling PSM and protein FDR at 1% and performing protein inference on the combined search results (415’201 peptide precursors, 12’381 protein groups). For comparison, a second version of the spectral library was generated where, for peptide precursors identified from both sources (DDA & DIA), the iRT as determined on the DIA runs was prioritized. These two spectral libraries were applied to the six DIA runs using the Spectronaut software (Biognosys) with a 1% peptide and protein FDR.

On the data set studied prioritizing iRT resulted in 15% more peptide precursor identifications (from 109’420 to 125’275). The cumulative number of identified protein groups in the six runs went up from 7’547 to 7’641. Additionally, also the number of consistently quantified peptides and proteins in all six runs increased.

Prioritizing iRT from data set sources closer to the setup used for the quantification increases coverage and reproducibility of quantification. In the future, this could be further improved by performing a tiered iRT calibration in Spectronaut when analyzing DIA data.

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Proteomic analysis of Neutrophil Extracellular Traps (NETs) produced in response to PMA and A23187

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Background

Neutrophil extracellular traps (NETs) are chromatin structures covered in anti-microbial proteins that are released by neutrophils in response to infection. The exposure of proteins and DNA within NETs may also trigger auto-immune responses, e.g. in systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), where auto-antibodies to double-stranded DNA (SLE) and citrullinated proteins (RA) may contribute to disease pathology. The aim of this work was to develop protocols for the measurement of NET proteins using quantitative proteomics.

Methods

Neutrophils were isolated from healthy individuals (n=3), and incubated +/- PMA (50nM, activator of PKC) or A23187 calcium ionophore (3.8µM, activator of PAD4) for 4h. DNA was digested. NET proteins were concentrated onto Strataclean beads and digested on-bead with trypsin. Peptides were resolved on 1h gradients. Data-dependent LC-MSMS analyses were conducted on a QExactive HF quadrupole-Orbitrap mass spectrometer. Label-free protein quantification was carried out using Progenesis QI.

Results

112 proteins, excluding those in culture media (+2% serum), were differentially expressed between conditions: untreated, PMA and A23187 ($p<0.05$, fold change >2). Nine proteins were significantly higher in PMA-treated compared to A23187-treated, including CYP4F3 (12.9-fold), Annexin A6 (4.9-fold), Major vault protein (2.8-fold) and Histone H2A type 2-B (2.3-fold). Fifty-five proteins were significantly higher in A23187-treated NETs compared to PMA-treated NETs, including Histone H1.0 and Histone H1.5 (26.7- and 21.4-fold), High mobility group protein B2 (13.0-fold), PAD4 (7.8-fold), Leukocyte elastase inhibitor (4.9-fold) and Catalase (3.2-fold).

Conclusions

PMA- and A23187-stimulated NETs released from healthy neutrophils were decorated with different proteins. Validation of these newly discovered NET proteins will be carried out by immunofluorescence. Future experiments will carry out comprehensive, quantitative analysis of the proteins decorating SLE and RA NETs to determine the molecular properties of NETs generated under auto-immune conditions.

Keywords

Neutrophil Extracellular Trap, NET, PMA, A23187

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Evaluation of Plasma Extracellular Vesicles Isolation Protocols for Proteome Analysis

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Background

Extracellular vesicles (EVs) represent an attractive source of possible diagnostic and prognostic biomarkers in several diseases. The high purity with high yield and less time-consuming EVs isolation protocol is needed for plasma EVs biomarker discovery. The ultracentrifugation (UC) has been widely used as a reliable protocol for isolating EVs with high purity, however, repeated UC steps are extremely time-consuming and reduce the yield of EVs. Recently, a number of commercial kits have been launched to overcome these problems. Several studies on comparing EV isolation kits and UC have been previously reported, but there is limited information regarding proteome analysis. In this study, we provide a comparative analysis of five EV isolation protocols for human plasma with high-resolution proteome analysis

Methods

A total of 100 µl pooled human plasma (CosmoBio) was used for EV isolation. Five EV isolation protocols including ultracentrifugation with sucrose cushion (UC), ExoQuick Plasma prep and Exosome precipitation kit (EQ) (System Biosciences), MagCapture Exosome Isolation Kit PS (MC) (Wako), Exosome Isolation Kit Pan, human (MB) (Miltenyi Biotec) and a hybrid method combined with UC and EQ (HY) were tested. Proteins in EVs isolated by these protocols were digested and analyzed by label-free quantitative method using LC-MS/MS (Q Exactive, Thermo Fisher).

Results & Conclusion

The number of identified proteins was 445 for UC, 337for EQ, 397 for MC, 312 for MB and 377 for HY. The purity of the samples was evaluated by relative quantitation of exosomal markers such as CD9, CD63, CD81, syntenin-1 and TSG101. Our date will help researchers in selecting the best EV isolation protocol for proteome analysis.

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A feasibility study of pitavastatin distribution in liver tissue by MALDI-Mass Spectrometry Imaging

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Background
In pharmacokinetic/dynamic studies, in vivo drug uptake into liver tissue is one important factor. For some drugs, transporters play an important role in their distribution in the liver. For further understanding of the in vivo drug disposition, it would be highly valuable to observe the drug distribution in the liver tissues with high image resolution. Statins are highly dependent on transporters for their uptake into the liver as well as further metabolism/excretion, and they are therefore interesting tool compounds for drug imaging. MALDI-MS imaging (MALDI-MSI) is the powerful technology to observe the drug distribution with high image resolution. MALDI-MSI can display with 30 µm image resolutions. In addition, it can evaluate the quantification of drug concentration, by making calibration curve with highly positive correlation co-efficiency.
In this study, we investigated the feasibility of statin distribution studies using MALDI-MS Imaging.

Methods
A portion (0.3 µL) of pitavastatin solution was dropped on a surface of rat liver tissue. For MALDI matrix deposition, the matrix was mixed with internal standard and sprayed with TM sprayer (HTX technologies, Texas, USA). MALDI-LTQ Orbitrap XL Mass spectrometer (Thermo Scientific, Bremen, Germany) was used for determination of pitavastatin distribution. Data analysis for pitavastatin quantification was conducted with the Image Quest (Thermo). These experiments were performed in triplicate.

Results
The range of pitavastatin concentration from 1 µM to 100 µM was investigated. The correlation coefficient (r) was 0.96-0.99 in this range. The detection limit was 1.5 pmol/spot (5 µM). According to the previous researches in in vivo studies, the pitavastatin concentration in liver tissue may reach 10 µM.

Conclusions
This work demonstrated the application that will be feasible with pitavastatin administration for rat model.

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Mass Spectrometry Histochemistry unveils hidden treasures in formalin fixed paraffin embedded biobanked tissue.

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We have established a protocol which enables the top-down analysis of a biologically highly relevant subset of the (human) (secreto-) proteome out of biobanked clinical material which is stored in paraffin blocks after standardized formalin fixation and embedding.

The method allows e.g. the successful mass spectrometry imaging of neuropeptides, which could previously only be detected when analyzing either fresh or freshly frozen neurosecretory tissue. Proof-of-concept was achieved employing a well studied model system, i.e., the cockroach pituitary equivalent. Actual studies of human tissues were performed on especially prepared FFPE tissue microarrays, containing a variety of different clinical biopsies, as well as ('healthy') control tissues including conventional histological sections through neuro- and adenohypophysis, and Homo sapiens cell lines.

The technique we developed is fully top-down, not employing trypsin or any other enzyme for protein identification purposes. As such the technique goes beyond mere protein or gene identification, but identifies the actual 'proteoforms'. Whereas it is obvious that the data obtained from high performance mass spectrometers have superior quality when compared to less performant systems, we would like to emphasize that the method principally is independent of the actual mass spectrometer employed. The method seems to work best with a MALDI source, although it is currently being optimized for SIMS systems as well.
Our approach has an evident bias towards small proteoforms/(endogenous) peptides of the proteome/secretome. Yet the obtained proteoform profiles represent unique 'bar codes' which are tissue and disease specific.

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Cell Wall Enrichment Reveals Further Dysregulation in Response to sub-MIC Rifampicin Stress

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Background
Tuberculosis disease is caused by Mycobacterium tuberculosis (M. tb) and is one of the leading causes of mortality globally. Mycobacterial drug resistance is a growing concern with frontline drugs, such as rifampicin, in danger of becoming ineffective. The cell wall of M. tb represents the point of contact between host and bacterium and understanding this complex functional barrier represents one of the most important tasks necessary for designing drugs and identifying vaccine targets. Additionally, we previously observed indications of dysregulation in the cell wall proteome of Mycobacterium smegmatis following exposure to sub-MIC concentrations of rifampicin, but without specifically enriching for the cell wall. In this study, we employed two gel-free approaches to enrich for cell wall proteins in M. smegmatis following exposure to sub-MIC rifampicin.

Methods
Mycobacterium smegmatis was treated with half-MIC of rifampicin and 255 minutes later was harvested and lysed by sonication. Proteins were purified by chloroform/methanol precipitation and the cell wall by differential centrifugation and solubilisation with detergent. Protein was prepared for proteomic analysis through in-solution digestion and desalting by STAGE tips. Analysis was by RP-HPLC-MS/MS using a Q-Exactive. MaxQuant was used for identifications and custom R scripts for statistical treatment and GO term enrichment.

Results
Dysregulation was observed in major cell wall transport proteins like TatB and many ABC transporters. Virulence factors such as MCE proteins and PknG were also dysregulated as were lipid synthesis and cell wall maintenance proteins such as FbpA and PimB.

Conclusions
The genuine cell wall was successfully enriched by these methods. Dysregulation in homologues of major mycobacterial virulence factors and other important cell wall proteins was here evident whilst lacking in our previous study that did not enrich for the cell wall proteome.

Keywords
Cell wall; Tuberculosis; Mycobacterium smegmatis; Proteomics; Rifampicin

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Improved Top-Down Analysis on an Orbitrap Fusion Lumos with the Advanced Precursor Determination Algorithm

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Background: Top-down data-dependent analysis workflows are typically plagued by repeated interrogation of different charge states of the same protein precursor. Through the development of an improved precursor charge state determination algorithm that works with both high and low resolution Orbitrap data, we are able to better associate all the ions representing a single proteoform in real-time. This added information improves the data-dependent decisions during the workflow, which in-turn maximizes the depth of analysis. We demonstrate the impact of this new algorithm on top-down analysis using an Orbitrap Fusion Lumos.
Methods: The Pierce intact protein standard mixture and an ecoli cell lystate were analyzed by top-down data dependent analysis using a high flow Dionex U3000 HPLC and an Orbitrap Fusion Lumos with Advanced Precursor Determination. Data dependent parameters were optimized to minimize redundant sampling of each protein and to improve sampling depth in both the 'low-high' and the 'high-high' modes of operation.
Results: We demonstrate here a significant improvement in sampling depth during data-dependent analysis of intact protein samples. The improved sampling depth is the result of the improved charge state determination algorithm, in conjunction with the option to perform dependent analysis on single charge state per precursor. Together these options greatly reduced redundant MS2 triggering on a given proteome, which allows for MS2 triggering on, and identification of, more unique analytes.
Conclusions: A new Orbitrap charge determination algorithm significantly improves depth of top-down analysis.
Keywords: Top-down, data-dependent, Orbitrap

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Development of a novel LC concept for clinical proteomics

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Background
Mass spectrometry based proteomics and metabolomics are fast growing and powerful technologies, with the potential to revolutionize health care and precision medicine. However, available separation technology has so far limited throughput and robustness and thereby prevented omics technologies from being fully integrated and routinely used in a clinical setting. Here we describe a conceptually novel chromatography system that significantly increases robustness and sample throughput while maintaining the sensitivity of current nano-flow LC.

Methods
The new system uses four low-pressure pumps in parallel to elute samples from a disposable trap column while also forming a chromatographic gradient. The sample and gradient are moved into a holding loop that subsequently is switched in-line with a single high pressure pump and a separation column. Thus elution from the disposable trap column and gradient formation become de-coupled from the high-pressure separation.

Results
We have characterized the performance of the new system regarding cross contaminations (<0.03%), retention time shifts and peak width (<8.5 sec) in over 1,000 HeLa runs. The short overhead time of approximately 2 min allowed us to measure 60 samples per day (22 min gradient, 2 min overhead time) compared to 40 samples with a standard nano-LC systems (22 min gradient, 15 min overhead time). The 1,000 human plasma samples were analyzed without any LC issues and each resulted in several hundred quantified plasma proteins, including more than 50 FDA-approved biomarkers with high reproducibility.

Conclusion
We have designed a new separation technology for LC-MS applications with the sensitivity, robustness and throughput required for clinical workflows and that will allow uninterrupted analysis of thousands of clinically relevant samples for biomarker discovery studies as well as for analysis of patient samples in clinical laboratories.

Keywords
HPLC, separation, clinical proteomics, LC-MS, biomarker

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An image-based subcellular map of the human proteome

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Background
Compartmentalization of biological reactions is an important mechanism to allow multiple cellular reactions to occur in parallel. Resolving the spatial distribution of the human proteome at subcellular level increases our understanding of human biology and disease. A high-resolution map of the human cell has been generated, the Cell Atlas, part of the open-access Human Protein Atlas.

Methods
Proteins were localized in human cell lines using an antibody and imaging-based approach. including strict validation criteria using gene silencing, paired antibodies, and fluorescently tagged proteins. Deep learning approaches and a citizen science approach was employed for refined pattern recognition in images, the mini-game “Project Discovery” integrated into an massively-multiplayer online game has engaged more than 200,000 players world-wide.

Results
A subcellular map of the human proteome has been created, comprising 12,003 proteins localized to 32 subcellular structures, enabling the definition of 13 major organelle proteomes. The high spatial resolution allowed identification of novel protein components of fine structures such as the midbody and nuclear bodies. We show that half of all proteins localize to multiple compartments. On one level, it can reflect a spatial confinement to control the timing of the molecular function in one compartment. On another level, such proteins may have context specific functions and ‘moonlight’ in different parts of the cell, thus increasing the functionality of the proteome and the complexity of the cell from a systems perspective. We further reveal 16% of the proteome to show single cell expression variation in terms of protein abundance or spatial distribution, and that current protein-protein network models benefit from integration of the Cell Atlas localization data as spatial boundaries.

Conclusions
We present the most comprehensive subcellular map of the human proteome and discuss the importance of spatial proteomics for single cell biology.

Keyword
Antibody, Cell Atlas, Image, Organelle, Citizen Science

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Comparative, LFQ-based proteomic analysis of growth-phase dependent changes of Lactobacillus kunkeei isolates from honeybee

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The gut microbiota of the honeybees is thought to be beneficial for the fitness and survival of bee colonies. It also serves a role as a model system for the human gut microbiota. For these reasons, a better understanding of the honeybee gut microbiota is of general importance.

Lactobacillus kunkeei is the most abundant bacterial species in the honey crop. We have isolated novel strains from two Scandinavian islands (Åland and Gotland). By using comparative genomic and proteomic approaches we are currently investigating the functional diversification of the bacterial symbionts. For the proteomic analysis, strains from Åland (A) and strains from Gotland (G) have been subjected to LC-MS/MS-based label-free quantification analysis to investigate growth-phase dependent changes (logarithmic versus stationary).

Overall, both the number of differentially expressed proteins and the levels of differential expression differed in these strains. Functional annotation of differentially expressed proteins based on COG categories showed relative differences in transcription/translation related proteins compared to metabolic proteins. These differences in growth-phase dependent changes on the proteome level might have physiological implications for the symbiotic lifestyle of the Lactobacillus kunkeei strains and potentially reflect differences in geographic origin, evolutionary history or bee genetics. To investigate these questions in more detail, we are currently expanding the proteomic analysis to a larger list of bacterial strains that belong to different phylogroups and have been isolated from different bee strains at different geographic locations.

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Molecular pathways impairment in Zika virus-infected neural stem cells revealed by proteomic approach

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Background
Zika Virus (ZIKV) is a flavivirus first isolated in 1947 in the ZIKA Forest in Uganda. Number of newborns with congenital malformation gave attention to new viral infection characteristics, which comprises congenital malformation spectrum including microcephaly. Indeed, neural progenitor cells (NPCs) seem to be the main affected population by ZIKV with consequent apoptosis, cell cycle arrest, early differentiation and flawed cell division, having as consequence depletion of neural progenitors. Regardless of recent reports on ZIKV effect on neuronal cells, pathways associated with physiopathology of the disease promoted by different strains are not completely understood.
Methods
MS-based quantitative proteomics approach was applied to provide a better understanding of the effect of ZIKV infection. Induced pluripotent stem cell-derived NPCs exposed to ZIKV-AF, ZIKV-BR or not, were cultured as neurospheres and used as 3D models to access the effects of ZIKV infection during neurogenesis. Peptides were TMT labeled and fractionated using HILIC prior to MS analysis.
Results
Using this strategy, we were able to quantify 4579 proteins with two or more peptides based on TMT reporter ion intensity of each labeled sample. Relative quantification comparison of proteins identified with two or more peptides showed 935 regulated proteins among NS MOCK, NS ZIKV-BR and NS ZIKV-AF. Focusing on the effect of ZIKV-BR on neurospheres, 897 proteins were found regulated, being 466 up-regulated and 431 downregulated. Pathway prediction analysis showed “Neurological Disease”, “Skeletal and Muscular Disorder”, “Cell Death and Survival” and “Embryonic Development” among overrepresented annotations (pValue <0.05, B-H FRD 5%).
Conclusions
ZIKV infection promotes overall expression deregulation on infected neurospheres. Host homeostasis is disturbed beyond cell cycle regulation, immune response or cell death. Besides previous reported reduced cell number, we have detected impairment of pathways related to neuronal function that could contribute for the observed phenotype.

Keywords
Zika virus; Neurodevelopment; Proteomics

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BayesProt: Robust iTraq and TMT protein-level quantification and statistical analysis with a Bayesian mixed-effects model

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Background: Proteomic biomarker discovery and downstream pathway analyses are reliant on statistically rigorous protein quantification and differential analysis. Experimentalists are now performing more complex studies with multiple conditions and repeated measures such as timecourses. Statistical packages must be flexible enough to model these, hence the success of tools such as MSstats that adopt the mixed-effects modelling paradigm.
Methods: We have recently presented a Bayesian approach for iTraq that infers relative protein-level quantification across samples together with the variance of each constituent peptide away from this pattern [Freeman et al. Diabetes 2016]. This key novelty enables robust protein-level differential analysis based on the most consistent quantification pattern reported at the peptide level. The model uses Bayesian MCMC simulation to assess the full range of plausible fold changes, outputing false discovery rate controlled probabilities for each protein. It separately estimates biological variation at the protein-level, technical variation due to peptide digestion, and residual variation at the spectrum-level (e.g. interferences). Multiple iTRAQ or TMT runs can be combined without a reference channel.
Results: We present an R and HTCondor-based software package ‘BayesProt’ for iTraq and TMT proteomics. BayesProt takes in the exported spectrum-level output of ProteinPilot (SCIEX) or Proteome Discoverer (Thermo), plus an Excel spreadsheet describing the experimental design, and outputs FDR-ranked protein-level fold-changes as CSV files. Due to BayesProt’s ability to determine unreliable peptides, quantitative validation on a spike-in study showed an 80% reduction in false positives compared to a conventional approach of performing statistical tests on the vendor software’s protein-level output. Since our approach establishes variability at the normalisation, peptide and protein-level, useful quality-control plots are also produced.
Conclusions: BayesTraq performs protein-level statistical analysis on a wide range of experimental designs, and in particular provides a rigorous method to determine the most quantitative peptides for each protein.

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MitoModules as cancer biomarkers: A technique to identify context-based protein biomarkers in mouse liver cancer

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Background: To date, most analyses for biomarker studies have been exclusively limited to protein abundance quantification treating each protein as an independent entity. In reality, proteins generally function in association with other proteins that are localized in specific subcellular compartments. We hypothesize that the subcellular localization and composition of protein complexes provides important information for the classification of clinical samples, which is not apparent from conventional protein measurements. To challenge our hypothesis, we generated chemically-induced mouse liver cancer for wild type and liver receptor homolog 1 (LRH-1) knock-out mice and performed statistical analysis on cell lysate and mitochondria-enriched fractions. To detect the biomarkers that differ between these conditions we used conventional protein data obtained from enriched mitochondria and total cellular lysates, as well as a subcellular localization enrichment scores (ES).

Methods: Targeted extraction of SWATH-MS acquired data was performed with OpenSWATH tool and spectral library of 3500 mouse proteins. In addition to protein expression levels measured both in mitochondria and cell lysate, we also considered a mitochondrial ES which were calculated from the expression data (i.e.ES=Log2(Mito)-Log2(Lysate).

Results: By using the conventional data from both sample preparations, we were able to detect the subset of liver proteome that changed significantly between the conditions. We found that ES were particularly informative about the cancer. ES did provide specific and readily interpretable biological information about subcellular localization of relevant factors. Moreover, unsupervised clustering from liver samples based on the ES revealed main clusters that were driven by cancer state and not by genotypes, suggesting that the combination of abundance and cellular localization information has high discriminant power.

Conclusions: Our data indicates that information about subcellular localization contains biological information that is not apparent from individual proteins.The method we developed is generally usable and can be extended to other fractionation techniques.

Cancer, Mitochondrion, SWATH-MS

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Extracellular Matrix Proteomics Reveals Aggrecan and Aggrecanases as Novel Contributors to Vascular Remodeling after Stenting

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Background: Extracellular matrix (ECM) remodeling contributes to in-stent restenosis and thrombosis. Despite its important clinical implications little is known about ECM changes post-stent implantation.

Methods: Bare-metal (BMS) and drug-eluting stents (DES) were implanted in pig coronary arteries with an overstretch under optical coherence tomography guidance. Stented segments were harvested 1, 3, 7, 14 and 28 days post-stenting for proteomics analysis of the media and neointima.

Results: A total of 151 ECM and ECM-associated proteins were identified by mass spectrometry. After stent implantation, the earliest changes in the media were proteins involved in inflammation and thrombosis, followed by changes in regulatory ECM proteins. By day 28, basement membrane proteins were reduced in DES compared with BMS. In contrast, the large aggregating proteoglycan aggrecan was increased. Aggrecanases of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family contribute to the catabolism of vascular proteoglycans. An increase in ADAMTS-specific aggrecan fragments was accompanied by a notable shift from ADAMTS1 and ADAMTS5 to ADAMTS4 gene expression after stent implantation. Immunostained human stented coronary arteries confirmed the presence of aggrecan and aggrecan fragments. Investigation of aggrecan presence in human vasculature revealed that aggrecan and aggrecan cleavage were more abundant in arteries compared to veins. Also, aggrecan synthesis was induced upon grafting a vein into the arterial circulation, suggesting an important role for aggrecan in vascular plasticity. Finally, lack of ADAMTS-5 activity in mice resulted in an accumulation of aggrecan in the aorta.

Conclusions: Coronary artery BMS and DES implantation lead to an up regulation of aggrecan, a major ECM component of cartilaginous tissues that confers resistance to compression. The accumulation of aggrecan coincided with a shift in ADAMTS gene expression. This study provides the first evidence implicating aggrecan and aggrecanases in the vascular injury response after stenting.

Key Words:
Extracellular matrix,stent,neointima,coronary artery disease,mass spectrometry

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Large-Scale Discovery and Quantitation of Novel Peptides using Omics Derived Libraries

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Background
Despite the recent advances in Data-independent acquisition Mass Spectrometry (DIA-MS), it is still constrained by the completeness and depth of the peptide assay library meaning that novel peptides or proteoforms may remain unidentified. Utilizing high-throughput RNA-Sequencing data to derive sample-specific assay libraries for DIA-MS can reveal novel single amino acid polymorphisms (SAPs), alternative splice forms and deeper proteome coverage.
Methods
The proposed workflow first detects alternatively spliced transcripts by aligning RNA-Seq data to retrieve coordinates of detected junctions. Next, the genomic sequences for these junctions are retrieved followed by a three-frame translation to produce polypeptide sequences. To identify these sequences using DIA-MS, we create an in-silico database of these peptides and perform targeted analysis using PECAN (Ting SY et al. 2014). Lastly, we manually verified and annotated the novel peptides to their respective gene families along with the genomic classification of the translation event.
Results
We used the GeneSTAR Study to profile the transcriptome and proteome of ~100 human megakaryocyte samples and generated a novel peptide database comprising of >100,000 splice-junction peptides. The targeted detection and quantitation of these peptides not only improved the proteome coverage but also identified potentially novel peptide translations of annotated proteins. We found several novel peptides for key platelet biomarkers such as Homeobox protein Meis1 required for hematopoiesis and megakaryocyte lineage development as well as Neurobeachin-like protein 2 involved in thrombopoiesis and platelet biogenesis. Quantitative validation of these targets using Parallel Reaction Monitoring experiments are in progress as part of the future validation efforts.
Conclusions
These data demonstrate that incorporating RNA-Seq data can enhance proteomics analyses for the discovery and quantitation of novel proteoforms. This parallel workflow will enable us to better understand novel risk assessment paradigms and identify new therapeutic targets for cardiovascular and thrombotic disorders.
Keywords
Novel peptides, RNA-Sequencing, DIA-MS, PECAN

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Secretome analysis of U937 DC-SIGN cell line after dengue infection

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Dengue is an important and growing public health problem worldwide with an estimate of 136 million new cases annually. Infection with dengue virus (DENV) results in a diverse range of symptoms, from mild undifferentiated fever to life-threatening hemorrhagic fever and shock. Further investigation on dengue pathogenesis is required to understand the reasons why some patients do not have symptoms while others develop the severe forms.
The principal target cells for DENV infection are monocytes, macrophages and dendritic cells, these cells recognize dengue virus infection with their pattern recognition receptors, and are involved in the activation of proper innate immune response. Proteins secreted by immune cells are able to modify a host's environment and modulate their immune system. Consequently, the secretome provides a promising resource for discovery of specific molecular markers and targets for pharmacological intervention.
Here, we employ label-free quantitative proteomics to characterize global protein secretion in DENV-infected U937 DC-SIGN cell line compared with mock-infected at different times post-infection. The quantitative profiles of macrophage secretome reveal the highly stimulus-dependent cellular response and differential, specific secretion of more than 250 proteins, including important proinflammatory proteins and cytokines.

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Proteomics of post-translational lysine acetylation in glioma-derived stem cells

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Background
Lysine acetylation is an important post-translational modification for the regulation of cellular processes, especially in transcription in nucleus. Recent proteomics studies showed this modification occurs in every cellular component including cytoplasm and mitochondria. Lysine acetylation has been shown to be associated with various diseases including cancer. Malignant glioma is the most common brain tumors with limited effective treatment options. Glioma-derived stem cells (GSCs) have been considered responsible for the therapeutic resistance and recurrence of glioma. Although HDAC inhibitors, which inhibit lysine deacetylases, are on the clinical trial as cancer drug, lysine acetylation of GSC/glioma cell (GC) proteome remains unknown. Here, we determined lysine acetylation sites in GCs and GSCs on a proteome-wide scale, and showed differences through bioinformatics analysis.

Methods
Proteins from GSCs and its differentiated GCs were digested by Lys-C and trypsin. Acetyl-peptides were enriched from the digests by immunoprecipitation using anti-acetyl-lysine antibodies. The enriched acetyl-peptides were analyzed by 2 h nanoflow liquid chromatography tandem mass spectrometry gradients on an Orbitrap Fusion mass spectrometer.

Results
We established the protocols of the cell culture, protein extraction, and peptide preparation from GSC spheres and differentiated GCs for the immunopurification of acetyl-peptides. The immunoprecipitated acetyl-peptides were analyzed by nLC-MS/MS. Hundreds of acetyl-peptides from both GSC and GC proteins located in every cellular components were identified. Interestingly, the number of acetylation sites and acetyl-proteins in GSCs were approximately 1.5-fold more than in GCs, suggesting the change of acetylation states during GSC differentiation. Furthermore, bioinfomatics analysis indicated that acetyl-proteins in cytoplasm, nucleus, and membrane were observed more often in GSCs than GCs. Detailed molecular analyses are under study.

Conclusions
Our acetyl-proteome study showed lysine acetylation occurring in GSCs and GCs for the first time. Further study will expand the knowledge of GSCs and their clinical targets.

Keywords
Lysine acetylation, Glioma, Glioma stem cell

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Accessing archival, formalin-fixed, paraffin-embedded tissue sample resources for proteomic analysis via PCT-SWATH

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Accessing archival, formalin-fixed, paraffin-embedded tissue sample resources for proteomic analysis via PCT-SWATH
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The mathematical association of molecular patterns generated by increasingly powerful genomic, proteomic or metabolomic techniques with the (clinical) phenotypes of sample cohorts is a powerful and promising strategy in biomedical research. Formalin-fixed, paraffin-embedded (FFPE) tissue samples, annotated with phenotypic and clinical outcome data represent a rich and unique research resource for longitudinal and retrospective studies of this kind.

In this study, we demonstrate that small (about 300 µg per punch including wax) FFPE samples can be converted at a throughput of 24 samples per day into proteomic maps, from which at least three thousand proteins can be accurately quantified at a high degree of reproducibility. This was achieved by the development of a detergent-free, rapid sample preparation method based on the pressure cycling technology (PCT), and SWATH-MS. By comparing protein patterns from matching fresh frozen (FF) and FFPE samples from 24 prostate cancer resections of the ProCOC sample cohort, we furthermore demonstrate that the respective patterns are generally comparable and that the same biomarker proteins, specifically AGR2 and POSTN, which correlate with biochemical recurrence-free survival (RFS), are independently identified from either sample type. Surprisingly, the proteomic patterns derived from FFPE samples identified additional proteins including DCN, DES, CSK and ASRGL1, the expression of which correlate with RFS.

The significance of the presented work is the demonstration that quantitative proteome patterns can be generated from FFPE samples at high sample throughput and reproducibility, thus connecting this rich resource of well annotated historical clinical samples to protein profiling and biomarker discovery.

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Identification of protein targets in cerebral endothelial cells for brain arteriovenous malformation (AVMs) molecular therapies

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Background

To develop a new molecular targeted treatment for brain (AVMs), identification of membrane proteins that are localised on the AVM endothelium is crucial. Current treatment methods are surgery and radiosurgery. However, complete occlusion post radiosurgery are achieved within 3 years, while patient remain at risk of haemorrhage. This study aims to identify potential protein targets in AVM endothelial cells that discriminate these vessels from normal vessels; these proteins targets will be investigated for the molecular therapy of brain AVMs to promote rapid thrombosis after radiosurgery.

Methods

We employed in vitro biotinylation that we developed, and mass spectrometry to detect cell surface-exposed proteins in cultures of murine cerebral endothelial cells (bEnd.3). Two forms of mass spectrometry were applied (iTRAQ-MS and MSE) to identify and quantify membrane protein expression at various time-points following irradiation which simulates a radiosurgical treatment approach. Immunocytochemistry was used to confirm the expression of selected membrane proteins. ProteinPilot V4.0 software was used to analyse the iTRAQ-MS data and the MSE data was analysed using ProteinLynx Global Server (PLGS) version 2.5 software.

Results

The proteomics data revealed several differentially expressed membrane proteins between irradiated and non-irradiated cells at specific time points, e.g. PECAM-1, cadherin-5, PDI, EPCR and integrins. Immunocytochemistry data confirmed the expression of these proteins.

Conclusions

Cell surface protein biotinylation and proteomics analysis successfully identified membrane proteins from murine brain endothelial cells in response to irradiation. This work suggests potential target protein molecules for evaluation in animal models of brain-AVM.

Keywords 5: Endothelial cells, biotinylation, membrane proteins, irradiation, arteriovenous malformations

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Precise structural characterization of unsaturated lipids by 213 nm UV-Photodissociation MSn

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The disruption of lipid metabolism is associated with several severe diseases. Mass spectrometry is a powerful analytical tool to advance our knowledge of lipid structures and functions. However, due to the high level of complexity of lipids, tandem mass spectrometry (MS/MS) strategies for their detailed structural characterization must offer more capabilities with regards to their gas phase ion fragmentation behavior, including multistage MS/MS (i.e., MSn). Here we investigate the molecular structural information provided for unsaturated lipids using 213 nm UVPD-MS/MS and MSn on a modified Orbitrap Fusion Lumos Tribrid MS. 213 nm UVPD of gas-phase lipid ions resulted in similar fragmentation behavior to that observed by conventional collisional activation (CID and HCD), but also provided diagnostic ions indicative of the sites of unsaturation for alkyl, sphingosine and plasmalogen-ether double bonds, as well as other unique structurally diagnostic information that were not obtained by HCD or CID. The data indicated that this is a generic behavior of UVPD and that the dissociation does not rely on the type of double bond or on the identity of the lipid category, class or subclass. Furthermore, HCD-MS/MS followed by UPVD-MS3 was shown to successfully enable assignment of the sites of unsaturation in triacylglyceride lipids, as well as for determination of the linkage sites in branched chain fatty acid esters of hydroxy fatty acids. Finally, UPVD-MS/MS of doubly deprotonated lipids such as Cardiolipin was found to efficiently yield a singly charged radical anion analogous to nETD, which could be structurally interrogated using HCD-MS3. Thus, UVPD is demonstrated to be a new and unique accessible strategy for unambiguous characterization of various lipid classes and represents a major breakthrough for lipid research, with a variety of applications in drug and biomarker development.

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Pediatric inflammatory bowel disease characterization by proteomics of pre- and post-therapy colon biopsies

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Background: Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic diseases of the gastrointestinal tract that affect over 4 million people worldwide. Pediatric patients represent 10-25% of the IBD population, presenting with more aggressive disease than their adult counterparts. While the etiology of IBD is not fully understood, it is accepted that environmental and genetic factors contribute to a dysbiosis of the microbiome and a dysregulation of the host immune response. To characterize disease pathogenesis, proteomics and bioinformatics analyses of colon biopsies from IBD and non-IBD patients were performed.

Methods: Biopsies from inflamed and/or non-inflamed regions were obtained during endoscopy from 43 and 36 treatment-naïve pediatric CD and UC patients, respectively, and compared with those from 41 non-IBD pediatric patients. In addition, biopsies were analyzed from 44 of the IBD patients after therapeutic intervention. A SuperSILAC approach was utilized to perform quantitative analysis of homogenized tissues. Following LC-MSMS, data was analyzed by bioinformatics tools including Maxquant, Perseus, and STRING.

Results: Hierarchical clustering and principal component analyses revealed proteomic patterns that distinguished inflamed from non-inflamed tissue, independent of therapy. Gene ontology and KEGG analysis revealed that proteins downregulated in inflammation are associated with metabolism, whereas upregulated proteins contribute to protein processing. A comparison of pre- and post-therapy CD patients identified over 100 significantly different proteins between patients that responded and those that did not respond to therapy, including proteins not previously implicated in IBD.

Conclusions: This study is the first of this scope to evaluate the proteomes of pediatric patients before and after therapy, identifying pathways that are altered in disease. Moreover, by direct comparison of samples from individual patients before and after therapeutic intervention, new insight into disease pathogenesis has been obtained.

Keywords: Inflammatory bowel disease,

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Identification and quantification of host cell proteins (HCPs) in human plasma derived biotherapeutics

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Background
Residual host cell proteins (HCPs) are contaminants in biotherapeutics, which may pose safety or stability risks. HCPs are typically found at low levels in highly purified proteins, and need to be monitored per regulatory guidelines. Most biotherapeutics are recombinantly expressed in Chinese Hamster Ovary (CHO) cells, and thus the HCPs monitored in these samples originate from the CHO host cells or the media source used in cell culture (mostly bovine). Another category of biotherapeutics are those that are derived from human plasma. For human plasma-derived products, immunogenicity is not usually a concern, unless the HCPs are in modified form, such as aggregation or oxidation. Instead, the primary concern is the biological function of the HCPs. HCP detection and measuring is particularly challenging for plasma proteins, mainly due to the highly glycosylated proteins found in humans. Here, we present a mass spectrometry and analysis workflow to identify and quantify host cell proteins for plasma-derived products.

Methods
Beta-2-glycoprotein 1 was purified from donor human plasma and is used as a model system. We spiked known protein digest standards to trypsinized protein at 1:50 and 1:1000 ratios and injected to Waters Synapt G2s mass spectrometer in MSE mode.
Data analysis was performed using Byonic and Byologic software (Protein Metrics Inc). Briefly, peptides were identified by Byonic using a Uniprot-human protein database, and identified peptides and proteins were quantified using Byologic.

Results
Spiked standards as well as several plasma proteins were detected and quantified down to ppm levels using our HCP workflow. Several of the HCPs were glycoproteins with N-linked and O-linked glycans, successfully identified by Byonic.

Conclusions
Detecting and measuring host cell proteins at ppm levels, including glycoproteins derived from plasma, is feasible with today's mass spectrometers and analysis software.

Keywords
Host cell proteins, HCP, plasma, glycoprotein, glycopeptides

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Differential proteome and secretome analyses of iPS-derived neural differentiation to unravel schizophrenia features

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Background: Schizophrenia (SCZ) is a complex and very severe neuropsychiatric disorder, with a wide range of debilitating symptoms. Several aspects of its multifactorial complexity are still unknown, and some are believed to have a neurodevelopmental origin. Therefore, human induced pluripotent stem cells (iPSCs) technology is of great interest as a model to better understand the disorder. Differentiating patient-derived iPSCs into neural cells, from progenitors to cerebral organoids, could bring insights into those neurodevelopmental aspects. Here we evaluated the global differential expression of proteins on SCZ patient-derived neural cells and secretome, leading to an integrated view of protein expression during brain development. Methods: Neural cells were cultured to maintain progenitor features, as well as 2D and 3D differentiation medium for different time points during development. Secretome of 48h-conditioned progenitor medium were analysed. Cells and secretome were processed for protein extraction and digestion. Peptides were subjected to state-of-the-art bottom up shotgun proteomics in a two dimensional liquid chromatography coupled to mass spectrometry in HDMSE mode. A label-free approach was used for proteome quantitation. Results: Whole cells analysis identified about 4,000 proteins, out of those 150 were differentially regulated. Schizophrenia patient-derived cells present a downregulation of proteins associated with metabolic and cell cycle processes and upregulation of proteins associated with nervous system development, inflammation and catabolic processes. Phosphorylation of signalling proteins, such as PRXD2 and SOD1, reveals key components to understand metabolic unbalance in SCZ. Connecting with the secretome, which unravelled altered expression of receptor localization to synapse, brain development and key components of signal transduction. Conclusions: The study of patient-derived neural cells is important for disorders of developmental origin, such as schizophrenia. A better understanding of the global proteome profile of these cells and secretome could bring new insights into the underlying mechanisms of schizophrenia. Keywords: neuroproteomics, schizophrenia, organoids, pluripotent stem cells

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Evaluation of NCI-7 Cell Line Panel as new CPTAC CompRef Material

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Background: The Clinical Proteomic Analysis Consortium (CPTAC) seeks to improve our understanding of cancer via the integration of genomic information with tumor proteome characterization. To evaluate the performance of mass spectrometry analysis at Proteome Characterization Centers (PCCs), as well as monitor workflow performance, a comparative reference material ("CompRef") was generated using breast cancer tumor xenografts. Although xenograft models are highly representative of the tumor's heterogeneity and microenvironment, the derived tissue contains proteins from multiple species and sample yield can be limited. A CompRef material derived from cell culture models has the potential to address these drawbacks, however, a single cell line would be insufficent to comprehensively reflect the human cancer proteome. Thus, we examined the NCI-7 Cell Line Panel for use as a new CompRef material for CPTAC PCCs.

Methods: Using the publicly available CellMiner database, a bioinformatic analysis was performed to identify the minimal number of cell lines providing >90% coverage of the human genome. We evaluated sample preparation reproducibility, as well as coverage of the human proteome using quantitative proteomics.

Results: In our analysis, seven cell lines were identified as providing >90% coverage of the human genome – LC_NCI_H23, LE_RPMI_8226, BR_T47D, LC_A549, CO_COLO205, LC_NCI_H226, LE_CCRF_CEM. Evaluation of sample preparation reproducibility, revealed robust sample digestion at the protein level at various concentrations, with accurate quantitation regardless of sample aliquoting at the peptide or protein level. Next, we determined coverage of the human cancer proteome, identifying >10,000 proteins, which is comparable to previous identification numbers of the xenograft CompRef material comprised of both human and mouse proteins.

Conclusions: Our analysis revealed the NCI-7 Cell Line Panel can be utilized as a new CompRef material for assessing current CPTAC PCC analysis platforms, as well as for benchmarking the performance of proteomics laboratories worldwide.

Keywords: CPTAC, TMT, CompRef standard, Differential proteomics

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Natural Polysaccharide Peptide (PSP) Promotes an IFN and TLR4 Induced Anti-HIV Response in Human Monocytes

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There is an urgent need to identify compounds that can reduce human immunodeficiency virus (HIV-1) replication without producing adverse effects. Coriolus Versicolor's polysaccharide peptide (PSP) has been studied for its immune-building capabilities with no significant associated toxicity. This study sought to determine whether PSP triggers an early immune response through Toll-like receptor 4 (TLR4) and interferon (IFN) induced proteins, by targeting HIV infection. In vitro cytotoxicity and dose assays were applied to evaluate PSP anti-HIV-1 activity on human THP1 monocytes infected with HIV-1 and treated with 200 µg/ml PSP over a 6-day period. HIV-1 viral replication inhibition was determined by p24 ELISA assay and antiviral chemokines were analyzed using luminex multiplex assay. Surface TLR4 flow cytometry analysis and quantitative [tandem mass tag (TMT) isobaric labeling] proteomics studies were performed to understand the cellular and molecular mechanisms behind PSP HIV-1 inhibition. PSP was found to achieve a viral inhibition of 61% in THP1 cells. Further studies in PBMCs from healthy (infected ex-vivo) and HIV-1 infected donors demonstrated PSP capacity to reduce viral replication to 64.02% and 16.54%, respectively. Chemokines known to block HIV entry, such as RANTES, MIP-1α, MIP-1β and SDF-1α were upregulated together with the upregulation and activation of TLR4 in HIV-infected, PSP-treated cells. Inhibition of TLR4 led to antiviral chemokine downregulation and increased HIV-1 replication. TMT data revealed the upregulation of IF-induced proteins, such as MX2, APOBEC3g, TRIM5 and EIF2A2K. Additionally, IFN-associated transcription factors as NF-kB, IRF7 and IRF9 were significantly upregulated in PSP-treated cells. On the other hand, NF-kB inhibitor was downregulated in PSP-treated cells, highlighting NF-kB key role in the anti-HIV response. These findings demonstrate for the first time that PSP induces an anti-HIV activity mediated by TLR4 and IFN and open new therapeutic avenues for HIV disease. Keywords: HIV-1, TLR4, PSP, Monocytes, Coriolus versicolor

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Establishment of the hnRNP C1/C2 role on schizophrenia through protein interaction network mapping

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Background
Schizophrenia consists on a chronic psychiatric disorder reaching 1% of world population. It is a multifactorial disease resulting of environmental and genetic factors. The pathology molecular mechanisms are mostly unknown, therefore several methodologies such as GWAS, transcriptome and proteome has been used to study and better understand the molecular basis concerning schizophrenia.
We have shown that proteins from hnRNP class are differentially regulated on post mortem brain tissues from patients with schizophrenia. Moreover, we also showed differential regulation of hnRNPs in oligodendrocytes cell culture when treated with clozapin, a common psychotic used in schizophrenia treatment, pointing to a role of hnRNP in schizophrenia. In this work, we employed co-immunoprecipitation (COIP) of hnRNP C1/C2 to investigate its interactions partners and have a more comprehensive understanding of this protein role on molecular processes involving schizophrenia.
Methods
Protein extract was obtained from MO3.13 cell line. The COIP of hnRNP C1/C2 was performed using Thermo immunoprecipitation kit. After COIP, protein pull and control were analyzed by mass spectrometry on a Synapt G2-Si (Waters Co) employing HDMSE acquisition mode. Protein identification was performed using Protein Lynx Global Server 3.0 (Waters Co).
Results
We identified the hnRNP C1/C2 interaction with eight different hnRNPs. These interactions were already described on literature in pairs but never all in the same protein complex. All these proteins are involved in splicing processes. Moreover, we identified three other proteins that were already mentioned as part of splicing pathway but never described together.
Conclusions
As mentioned before hnRNP C1/C2 had been already described as unregulated on brain tissues from schizophrenia patients. In this way, the identification of its partners provides new insights about the molecular mechanism of this protein as well as a new perspective of proteins that could be involved in schizophrenia processes.
Keywords
hnRNP C1/C2, schizophrenia, protein interaction

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Middle-down analysis of monoclonal antibody with ETD and UVPD on an Orbitrap Fusion Lumos MS

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Sub-unit mass spectrometry analysis of monoclonal antibodies is a common assay although the fragmentation of sub-units (middle-down) to obtain crucial sequence information is still highly challenging. Currently, the preferred fragmentation technique for large molecules is ETD. Additionally, the possibility to use wide isolation windows and therefore isolating multiple charge states for fragmentation, and, the ability to control the duration of ETD reaction and AGC targets for both anions and cations are key factors that contribute to high fragmentation efficiency. Here we investigate the structural information of monoclonal antibody standards using UVPD and ETD LC/MS/MS. Trastuzumab and NIST mAbs were used as standards and were digested with IdeS followed by denaturation and reduction. LC/MS experiments were performed using Orbitrap Fusion Lumos Tribrid MS with a 213 nm UVPD source and coupled with a Vanquish UHPLC system. LC-MS raw files were processed with Biopharma Finder for sub-unit analyses. Targeted ETD and UVPD data were acquired and MS/MS spectra were deconvoluted using XTRACT. Finally ProSight Lite was used to generate matched fragment ions. Preliminary results showed that by combining different reaction times for ETD, up to 70% sequence coverage can be obtained for Trastuzumab and NIST domains (Fc, LC, Fd). Additionally, in two back to back ETD and UVPD LC/MS experiments, up to 80% sequence coverage was observed for the NIST sub-units where UVPD provided over 30% increase in sequence coverage.

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Implementation of 213 nm Ultra Violet Photo Dissociation (UVPD) on a modified Orbitrap Fusion Lumos

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Background - Ultra Violet Photo Dissociation (UVPD) at a variety of wavelengths has been demonstrated to be a useful tool for the analysis of peptides and proteins. This presentation explores the implementation of 213 nm UVPD on a modified Orbitrap Fusion Lumos instrument using a compact, robust, passively Q-switched, 2.5 kHz, 1.5 uJ/pulse solid state Nd:YAG laser. Details of the implementation are discussed and performance related to top down protein sequencing is evaluated.
Methods - The back flange of the dual cell 2D linear ion trap chamber of an Orbitrap Fusion Lumos instrument was modified to allow the laser and steering optics to be interfaced to the instrument in quite a compact configuration. The laser beam enters the chamber through this modified flange, and irradiates ions within the low pressure cell of the dual trap assembly. Laser triggering is controlled by the instrument scan function and ions are activated by a train of 2.5 kHz laser pulses for irradiation times which corresponds to a single laser pulse up to many thousands of laser pulses, while being stored in the low pressure cell. Mass analysis is achieved in either the ion trap or orbitrap mass analyzer.
Results - The performance of 213 nm UVPD with the solid state laser on the modified Orbitrap Fusion Lumos instrument is evaluated by measuring the sequence coverage obtained for apomyoglobin, and carbonic anhydrase. We show the evolution of the sequence coverage versus activation time, and compare the results of 213 nm UVPD to those obtained with other activation techniques including HCD and ETD.
Conclusions - The implementation of 213 nm UVPD on the OT Fusion Lumos is discussed and preliminary data regarding the utility of the technique with respect to top down sequencing is presented.

Keywords - 213 nm UVPD Fusion Lumos

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Fucosylated Glycoproteins Associated with Advanced Prostate Cancer

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Prostate cancer is the most common type of cancer found in men and androgen-deprivation therapy remains the principal treatment for patients with prostate cancer. Although a majority of patients initially respond to androgen-deprivation therapy, most will eventually develop to advanced cancer with castration resistance. An increased understanding of the mechanisms that underline the pathogenesis of castration resistance is therefore needed for novel therapeutics. Glycoproteins are most expressed on the extracellular space of cells and constitute the interface between the interior and the outside of cells. Aberrant protein glycosylation is known to be associated with the development of cancers. To determine the aberrant glycoproteins that are produced by the combined actions of changed protein expression and glycosylation synthesis pathways in prostate cancer, we analyzed the expression of glycoproteins and glycosyltransferase genes in advanced prostate cancer tissues and cell lines and found that the over expression of α (1,6) fucosyltransferase (FUT8) was statistically associated with advanced prostate cancer with a high Gleason score and in androgen-independent prostate cancer cells. We further found that FUT8 overexpression in LNCaP cells increased cell migration, while loss of FUT8 in PC3 cells decreased cell motility. We further developed method to quantitatively analyze fucosylated glycoproteins from intact glycopeptides and found that elevation of fucosylated glycoproteins in androgen-independent prostate cancer cell lines and metastatic castration-resistant prostate cancer tissues. Our results suggest that fucosylated glycoproteins may be associated with castration-resistant prostate cancer and thus is potentially useful for developing prostate cancer treatment options.

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Integrating simulated spectral libraries into proteomics data analysis workflow for improved phosphosite identification.

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Background
One of the main computational challenges in phosphoproteomics is how to determine the exact phosphorylation site(s). Earlier we proposed a method based on simulated phosphopeptide spectral libraries, which enables highly sensitive and accurate phosphosite assignments. Here we will evaluate possible downstream analysis strategies and pre-processing steps, necessary for data format compatibility.
Methods
Simulated phosphopeptide spectra were used as a reference in the spectral search of observed phosphopeptides. The simulation was done using the recently developed SimPhospho software tool, which was implemented in C++. The tool includes components of the Proteowizard project and a user interface based on the Qt framework. It is available for Windows, Linux and Mac operating systems. Spectral libraries were built and searched using SpectraST. For benchmarking, more than a hundred synthetic peptides were chosen to represent different isoforms of phosphopeptides with one and more phosphorylation sites. Phosphopeptides were pooled into three pools and analysed by Orbitrap HCD in Q Exactive.
Results
Comprehensively simulated phosphopeptide spectra by SimPhospho in combination with spectral library searching enables highly confident phosphosite validation. Spectral simulation is currently possible using results from Mascot and X!Tandem. The development of solutions for other input data is ongoing.
Conclusions
In this tutorial we will present a detailed description of a data analysis workflow using SimPhospho, focusing in particular on how it can be integrated with various software applications, such as Trans-Proteomic Pipeline, Proteome Discoverer and Peptizer. Potential and limitations of the existing approaches to phosphosite validation and identification will be presented, together with an overview of their performance.

Keywords
phosphosite, spectral libraries, phosphoproteomics

Characterisation and comparison of in-house produced Cetuximab with originator product

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Background

Cetuximab is one of the highest grossing and frequently prescribed monoclonal antibody treatments. The patent for Cetuximab is void in 2018 and biosimilars are coming in to late stage clinical trials. This study compares batches of originator product, produced in Sp2/0, with in-house produced Cetuximab. The CDR sequence of Cetuximab was cloned into a human IgG1 frame and transiently produced in HEK and CHO. Cation exchange chromatography using a pH gradient was used to determine the charge variant profile of each mAb, following on middle up MS, Bottom-up and Glycan profiling. This study provided a comprehensive and intriguing insight into the differences between in-house and originator mAbs.

Methods

Charge variant analysis performed on a Thermo Scientific Vanquish UHPLC, MabPac SCX with pH gradient buffers. Separation of IdeS digested mAb fragments performed on Thermo Scientific Vanquish RP-UHPLC. Peptide Mapping performed on a Thermo Scientific Q -Exactive BioPharma. N-glycan mapping was performed with PNGase F, 2-AA labelling separation on Thermo Scientific Vanquish UHPLC, Amide HILIC column. H218O labelling was performed with PNGase F digestion to determine site occupancy of glycan structures.

Results

Peptide mapping showed full sequence coverage of each of the Cetuximab variants. N-glycan profiles were observed to be distinct to each cell line. Analysis revealed lysine clipping as well as the presence of sialic acid containing glycans to be main causes for charge heterogeneity. H218O labelling of glycosylation sites enabled accurate identification of N-glycan site occupancy and deamidation occurring due to sample handling during de-glycosylation.

Conclusions

This study provides an interesting insight into full characterisation and comparison of a commercially available mAb with in-house produced mAb variants. There is also an interesting comparison between the human cell line produced and the CHO produced and the differences even this small difference can make to the overall composition of the product.

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Cardiotonic steroids reveal high interference on calcium–binding/dependent protein expression in H9c2 and Hek-293 cells

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Background - Calcium plays a very important role in the contraction of heart muscles. The cellular concentration of Ca2+ inside the cells is carefully controlled by Na+/K+-ATPase pumps, sodium-calcium exchangers and auxiliary proteins, including calcium-binding/dependent proteins. Hyperglycemia episodes in diabetic patients enhance the vascular contractility. Similarly, natural cardiac glycosides present in human serum also interfere with the cardiac action potential by blocking Na+/K+- ATPase pumps. However, the effect on the metabolic pathways of H9c2 and HEK cells when induced simultaneously or individually by hyperglycemia and by addition of cardiac glycosides is completely unknown at protein expression level. Therefore, the purpose of this work was simulating hyperglycemia episodes in the presence of digoxin and ouabain to verify the protein expression.
Methods- Protein extractions (all triplicates) were performed with Urea-thiourea. For protein digestion 1.0 mg of total protein were used for each treated and untreated conditions. Total proteins were reduced (DTT 50mM), alkylated (IAA 50 mM) and digested with trypsin. Samples were desalted (1/2 labelled with iTRAQ) and submitted to LC-MS/MS analysis in an Orbitrap Velos mass spectrometer. MS data were analyzed by Discoverer 2.0 program against Rattus norvergicus protein database. Subsequently, samples were analyzed by Scaffold and only proteins which change fold surpassing two times the areas of respective controls were considered as significant change expressions.
Results – Using iTRAQ labeling and label free methods more 1300 different proteins were identified and quantified. Dozens of Ca2+ binding/dependent proteins were also identified and quantified showing significant altered expression. Except alpha-actinin-1, annexin-1, annexin-2, calpastatin, calponin-1 and calponin-3 that increase expression when activated, all the rest Ca2+ binding/dependent proteins quantified decrease protein expression.
Conclusions – Activation of H9C2 and HEK cells with digoxin, ouabain and glucose interferes on protein expression.
Keywords – H9C2 cells, Ouabain/Digoxin, Hyperglicemia, Ca2+proteins, MS quantification

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Quantitative determination of specific serum IgG; diagnosis of Staphylococcus aureus bloodstream infection as an example

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Background
Staphylococcus aureus is a serious pathogen, which is responsible for many nosocomial infections. S. aureus persistently colonizes approximately 20-30% of the human population. Even if carriers have an increased risk of infection, the course of infection is less severe than in non-carriers. To elucidate if the specific adaptive immune response can provide protection in blood stream infection a case control study was performed.

Methods
FlexMap 3D measurements of plasma IgG binding to a panel of S. aureus proteins (n=64) were performed from serum samples at diagnosis of S. aureus bloodstream infections to explore if antibody titers are associated with outcome/protection. Analysis was performed with serum dilution series and a newly developed non-linear data analysis tool was applied to determine the specific IgG response levels of each patient.

Results
Analyzing seven different serum dilutions we were able to quantitatively resolve the antigen binding dynamics to S. aureus proteins. The specific signals highly varied between patients and bacterial antigens. In general, a more pronounced immune response was observed to extracellular or surface-associated compared to intracellular S. aureus proteins. S. aureus-specific IgG levels at diagnosis of infection seemed to be suitable to stratify patients into groups which subsequently developed sepsis or not. The specific IgG binding pattern of eight S. aureus proteins correctly predicted the disease course in 75% of the patients.

Conclusions
Serological assays carried out with dilution series have a higher resolution power than single dilution assays, especially if antibody binding varies over a broad range.
Seven of the eight predictive S. aureus antigens are conserved and belong to the S. aureus core genome, which indicates that they might be suitable candidates for diagnostic tools.

Keywords
xMAP technology, IgG response, sepsis, immune response, bloodstream infection

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MissingProteinPedia (MPP), a platform to uncover the human 'Missing proteins' (www.missingproteins.org)

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The Human Proteome Project (HPP) supports defining what it is to be human in molecular terms. It strives to "know thyself" by finding high-stringency evidence for the ~20,000 proteins encoded by the human genome. Here, we focus on what has been termed the human proteome's "missing proteins". Our re-analysis reveals a need for the community to capture as much complementary evidence as possible about missing proteins, in addition to high-stringency MS data. To do so, here we launch the MissingProteinPedia (missingproteins.org), a community biological database that is complementary to currently available high-stringency HPP methods. This feature rich evolving database has key features that allows for automated data collection, sharing and collaboration, integration of novel tools to assist annotation and discovery of missing proteins from user supplied datasets, and a publications viewer to enable the quick and effective searching of 'missing' proteins in the literature. MissingProteinPedia is a low-stringency communal database that will increase our understanding of the spatiotemporal biology of missing proteins, and accelerate their discovery by high-stringency MS.

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Investigating the potential of exosomes as biomarkers in Cystic Fibrosis

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Background
Mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene form the basis for the clinical manifestations of CF which affects the epithelial innate immune function in the lung. Epithelial dysfunction results in the initiation of a pro-inflammatory cascade in CF, the mechanisms of which are not clearly understood. Exosomes are nanovesicles (40–100nm) actively secreted by most living cells that contain proteins involved in cell-to-cell signalling within the extracellular microenvironment. We hypothesised that exosomes released from CF lung cells may play an important role in regulating immune cell migration and may have biomarker potential in CF.

Methods: Exosomes were isolated from WT- and ΔF508 bronchial cells and CF patient BAL fluid using ultracentrifugation. Exosome fractions were analysed by mass spectrometry. Bioinformatics using Ingenuity Pathway analysis was performed. Exosomes were further characterised by NanoSite NS300 analysis and biochemical assays.

Results: We observed a higher number of exosomes released from ΔF508-CFBE cells compared to WT cells using Nanosite NS300 analysis. There was also an increase in the number of proteins identified in ΔF508-derived exosome populations. Ingenuity pathway analysis showed that the top pathways that differ between exosomes from WT vs ΔF508 exosomes include integrin signalling and acute inflammatory response. We are currently characterizing the functional role of exosomes in CF in relation to regulating cell migration. Furthermore, we analysed exosome profiles from CF patient BAL fluid from adults and children with CF. We identified unique protein fingerprints between different patient populations and > 100 different proteins between BAL fluid isolated from CF patients and controls.
Keywords; Cystic Fibrosis, Exosomes, Proteomics, Biomarkers

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Serum fragment of TSP1 as a biomarker for metastasis and poor prognosis of ESCC

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Background Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant neoplasms in Asia. Patients are often diagnosed at advanced stages due to the absence of obvious early symptoms. Therefore, it's urgent to find early biomarkers of ESCC and understand their biological function, which may improve the ESCC treatment.
Methods Serum of ESCC patients (n=201) and healthy controls (n=196) were analyzed using a magnetic bead-based MALDI-TOF-MS assay, N-terminal fragment of TSP1, overexpressed in ESCC, was further studied. Above peptides was synthesized to generate a monoclonal antibody, which was subsequently used to construct a competitive ELISA assay to detect the above peptides in more patients. At the same time, small interfere RNA was use to alter the expression of TSP1 in ESCC cells, then wound healing assay and invasion assays on transwell plates was performed to evaluate the change of cell mortality.
Results A monoclonal antibody specific recognize the N-terminal fragment of TSP1 was generated, subsequent constructed ELISA assay showed the increase of TSP1 fragment in ESCC patients comparing with healthy controls ($P<0.005$). Knocking down of TSP1 significantly decreased the migration and invasion abilities of ESCC cells in vitro.
Conclusions We found that serum N-terminal fragment of TSP1 is overexpressed in ESCC patients and contributes to the migration and invasion in vitro. Therefore, TSP1 may be used as an potential circulating tumor marker and drug target to assist early diagnosis of ESCC and therapeutic monitoring.
Keywords: esophageal squamous cell carcinoma; MALDI-TOF-MS; TSP1; ELISA; invasion

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Quantitative Evaluation towards the Glutathione S-Transferases in Human Plasma Using Affinity coupling with LC-MS/MS

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Background
Glutathione S-transferases (GSTs) belong to a protein super-family which widely involves in many biological functions and plays as the indicators for pathological changes, such as carcinogenesis and drug resistance. Even though the traditional approach, ELISA or measurement of enzyme activity, could detect some GTSs, there is still lack of a globally quantitative evaluation towards GSTs. At omics era, we realize that a global evaluation is always helpful to provide overall information for such protein family members. Herein, we proposed a quantitative measurement to GSTs in plasma through combination of affinity chromatography and Parallel Reaction Monitoring (PRM).

Methods
GST-affinity chromatography was used for enrichment of GSTs in human plasma. Generally, 600µL plasma was incubated at room temperature for 2 hours with 30µL glutathione-resin coated on magnetic beads. The bound GSTs were digested on magnetic beads with trypsin. The tryptic peptides were delivered onto nanoLC for separation followed by target analysis on Q-Exactive HF™ at PRM mode.

Results
In the preliminary experiments, 103 GST peptides were selected as the candidates for PRM detection. We tested the GST binding efficiency using different volumes of affinity beads and found 30µL magnetic beads enough for targets enrichment with consistently identifying 77 GST peptides. Additionally, we found that desalting could highly improve the quantitative reproducibility of the GST peptides. Different volumes of plasma and optimized dilutions for GST enrichment were evaluated carefully, resulting in that 600µL plasma with one time diluted by the binding buffer could generate more stable PRM signals. With optimized conditions, 54 peptides (CV < 0.2) corresponding to the 20 different GSTs were detected with satisfactory MS/MS signals.

Conclusions
We have achieved a substantial progress to develop a LC-MS approach for global quantification of GSTs in human plasma.

Keywords
GST, target proteomics, affinity chromatography, LC-MS, PRM

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Unveiling the translational consequences of fusion genes and isoforms present in cancer using proteogenomics approach

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Background
Broadly, fusion protein encoded by combination of two genes results from chromosomal translocation or trans-splicing which often shows aberrant function due to its noncanonical sequence. However, there are a few reports on of their existence in protein level and no common bioinformatics tools are available in predicting their sequences, which is essential for studying their role in cancer microenvironment.
Methods
To generate sequence database of fusion products, we developed FP-Analyzer, a proteogenomics pipeline. This tool was designed to predict fusion genes and infer their isoforms using three well-known gene fusion finders and filter out false predictions. From the filtered results, three-frame translation module finally builds customized database of fusion ORFs. In this study, two breast cancer cell line (MCF-7, HeLa) and two leukemia cell line (K-562, Jurkat) data were used to identify and characterize fusion products specifically present in each sample by using this platform.
Results
FP-analyzer shows high sensitivity and low false discovery rate in terms of prediction performance. Our results obtained from FP-Analyzer suggested that over the half of the fusion transcripts use canonical splicing site to integrate two fusion partner gene sequences. Additionally, the definition of fusion product was extended to cover not only a combined sequence of two separated proteins but also a frame-shifted or truncated sequence of one fusion partner protein, which enables probe the evidence of fusion event in protein level. Our results revealed that many of expected sequences of fusion junctions are not covered by a tryptic peptide, suggesting the necessity of multi-protease MS/MS experiments.
Conclusions
We constructed an integrated pipeline, termed FP-analyzer, for predicting and analyzing fusion genes, transcripts and their products. To the best of our knowledge, it is the first attempt to obtain and analyze putative fusion transcript sequences and fusion ORF sequences.
Keywords
Proteogenomics, fusion protein, fusion ORF

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Plasma membrane proteomic analysis of host protein expression in response to Enterovirus 71 infection

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Background: Although it has been widely recognized that Enterovirus 71 (EV71) enters host cells through receptor-mediated pathway, the details of entry mechanism for EV71 are still unclear. The aim of this study is to investigate the host plasma membrane proteins involved in EV71 infection. Methods: Human Rhabdomyosarcoma (RD) cells were infected with EV71 for 2 hours. Plasma membrane (PM) of EV71 treated or non-treated RD cells were separated by two-phase system and the PM extraction effect was verified by western blot (WB). An iTRAQ based proteomic study was performed. Results: The plasma membrane marker protein was enriched for 4 fold in the PM fraction, and the mitochondrion contamination was decreased for 3 fold compared with homogenate. Through iTRAQ based proteomic study, 14 differentially expressed proteins with ≥ 1.5-fold change were detected, in which 7 proteins were up-regulated including BAG family molecular chaperone regulator 2, peroxiredoxin-2, and alpha-enolase (ENO1), and 7 proteins were down-regulated including heterogeneous nuclear ribonucleoproteins C1/C2 and endoplasmin, etc. 13 of these differential proteins have plasma membrane localization. These differently proteins were involved in enzyme and binding related functions. ENO1 was verified to be up-regulated by western immunoblotting. After ENO1 knockdown by ShRNA (ShENO1), the EV71 infection rate was decreased. ShENO1 was detected to have stronger inhibiting effect of EV71 infection compared to ShSCART 2 and ShAnnexin A2 (two known receptors of EV71). Furthermore, ENO1 was detected to be interacted with VP1, a structure protein of EV71, by immunoprecipitation experiment. Conclusion: An iTRAQ based proteomic study was performed and ENO1 was found to be related to EV71 infection. Knock down of ENO1 have stronger inhibition effect on EV71 infection compared to knockdown of known EV71 receptors. These findings provide new clues for understanding the molecular mechanism of EV71 entry and infection.Key words: EV71, plasma membrane, proteomics, iTRAQ, ENO1.

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Mechanistic insights into Ca2+ dependent regulation of cell cycle by label-free LC-MS/MS.

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Background:Capacitive calcium entry in the form of Ca2+ influx is known to supports growth factor induced G_0/G_1 transition and progression through G_1 to S phase. Although much is known of the molecular details of how the cell cycle is regulated, the mechanism that links Ca2+ influx and cell cycle progression has yet to be elucidated. Using semi-quantitative, label-free and unbiased proteomics we have identified proteins and pathways that are regulated by the inhibition of Ca2+ influx in growth factor stimulated human fibroblasts.
Methods:The effect of Ca2+ influx on protein expression during cell cycle was investigated in cells treated for 3h with growth factors in the presence or absence of Ca2+ influx inhibitor (SK&F96365). Cell lysate proteins were digested and peptides analysed on a Q-Exactive mass spectrometer. Label-free protein identification and quantification was performed using MaxQuant and Perseus software. Pathway analysis was performed using Ingenuity Pathway Analysis®. The protein expression data were validated by Western blot and functional assays.
Results:Using label-free mass spectrometry, almost 4,000 proteins were measured and of these the expression of around 170 was found to be altered by Ca2+ influx inhibition. Proteins that were regulated by Ca2+ influx were associated with the regulation of cell cycle or cell shape and some proteins such as ACP1, CDC42, HRAS, PXN, RHOB were found to be involved in regulation of both cell processes. Validation of selected proteins and selected biological processes was in agreement with data obtained by LC-MS/MS analysis.
Conclusions:The data show that Ca2+ influx regulates growth factor induced protein expression early in G_1. Importantly, proteins that are regulated by Ca2+ influx are involved in the maintenance of cell shape and cell proliferation. Pathway analysis has revealed potential mechanisms by which Ca2+ influx may regulate cell cycle progression and cell proliferation.
Keywords:cell cycle, G_1 phase, Ca2+ influx, label-free LC-MS/MS

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Online 2D-LC intact proteins separation for deeper shotgun proteomics of serum samples

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Background
The analysis of complex protein mixtures usually starts with their proteolytic digestion with known enzymes followed by nanoLC-MS. The proteome profiling of crude serum is a challenging task due to the wide dynamic range of protein concentrations and the presence of high-abundance proteins, which cover >90% of the total proteins in serum. Thus, the commonly used fractionation techniques on the peptide level normally have little impact on the deepness of serum proteome as peptides from high-abundant proteins are distributed over multiple fractions. The separation of intact proteins is more promising for serum proteome analysis. Here we describe an online 2D-RP/RP fractionation method that was used to dig deeper into human serum proteome.
Methods
The Agilent 1290 Infinity online 2D system was used for reversed-phase protein fraction at high pH in the first dimension and low pH in the second dimension. The automatically collected fractions after the 2nd dimension were digested with trypsin and analyzed with nanoLC-MS (UltiMate 3000 RSLCnano coupled to a Q Exactive Plus mass-spectrometer). The method was validated on 3 “pooled” depleted (64 most abundant proteins) serum samples from healthy subjects and 2 stages of cervical cancer patients.
Results
We have thoroughly optimized the RP separation of intact proteins at high and low pH using water/acetonitrile mobile phases. 2D RP/RP intact protein separation and fraction collection was accomplished in 120 min with 12 fractions transferred for separation in the 2nd dimension. More than 350 protein groups (<1% FDR) were identified in all depleted 3 sera samples after online 2D-LC pre-fractionation followed by nanoLC-MS/MS analysis. The dynamic range of identified proteins covered 4 orders of magnitude.
Conclusions
Online 2D LC separation of intact proteins with automated fraction collection allows looking deeper into the human serum proteome in view of biomarker discovery studies.
Keywords
2D-LC
Intact protein
Serum
NanoLC-MS

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Glycan profiling for exploring relationship of MUC1 O-glycoform with tumor-characteristic by lectin microarray

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Background; Clinical tissue sections provide useful information related to various tumor events. Therefore, these are attractive clinical resource for omics-based biomarker discovery including glycoproteomics. The quantitative analysis of O-glycoform, such as MUC1 of cell surface mucins, targeting to tissue sections is still difficult in terms of the sensitivity and throughput even if it is analyzed with current technologies. In this study, we demonstrated the differential glycan profiling for direct O-glycoform analysis of membrane-tethered MUC1 from slight tissue fragments with antibody-assisted lectin microarray (ALM).
Methods; Initially, a pair of serial formalin-fixed paraffin-embedded (FFPE) tissue sections of cholangiocarcinoma (CCA) and pancreatic ductal adenocarcinoma (PDAC) expressing MUC1 was prepared for the immunostain and tissue dissection. After the distribution of immunostain with MY.1E12 that was established as a monoclonal antibody recognizing MUC1 glycosylation isoform with a sialyl-core 1 structure (NueAcα2-3galactosylβ1-3-N-acetylgalactosamine), tissue fragments were obtained from the MUC1-positive area on FFPE tissue sections by laser capture microdissection. MUC1 was enriched by immunoprecipitation with MY.1E12 from the extracted glycoprotein lysate of the tissue fragments and then performed glycan profiling of the purified MUC1 with ALM.
Results; We obtained the glycan profile of the membrane-tethered MUC1 from tissue section of 2.5 mm2 in 5 µm thickness as MY.1E12-positive area. Differential glycan profiling between 21 CCA and 50 PDAC showed clearly the difference in the glycoforms on these MUC1, which were supported by lectin-MY.1E12 fluorescence double immunostaining. Moreover, we found specific lectin reflecting to the degree of tumor malignancy.
Conclusions; We established an effective method for O-glycoform analysis of MUC1 corresponding to the characteristics of tumor on FFPE tissue sections. Our approach is expected to facilitate the biomarker discovery associated with the glycosylation of mucins.
Keywords; MUC1, Glycan profiling, Glycoproteomics, Biomarker, Lectin microarray

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Towards minute-made MS assays using surface acoustic wave nebulisation (SAWN)

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SAWN is a recently developed ambient ionization method that generates a fine mist containing analytes amenable to direct mass spectrometric measurements. The technology platform offers competitive advantages over ESI and MALDI by reducing the analysis time and experimental complexity.

The SAWN device was placed in front of the API inlet of a triple TOF 5600+ MS and operated through tablet connected to a frequency and power controller. 1 uL of liquid sample is loaded on the chip and the SAW is regulated by applying a power to the electrodes (≈5 W). Depending on the sample, this step is repeated several times acquiring the data in continuous mode.

In a forensic study, we investigated a time-dependent signature of dried blood spots from different donors. The results from the 3 donors aged up to 8 days where tested for normality for both nanoLC-MS/MS and SAWN-MS. It was confirmed that there was a difference in total ion current traces between days and patient groups. Inter-day and inter-donor differences were observed for identified proteins and thus may lead to rapid time stamping for forensic blood samples. The obvious advantage of using SAWN-MS is a reduction in acquisition time from 90 to 2 min per sample, and spectral matching (min) of samples over cumbersome identification and comparison-studies (days).

The SAWN-MS technique has also been started for the analysis of sputum samples from cystic fibrosis (CF) patients. This study aims to discriminate between the presence of (or changes in) different microbial species, and to discriminate between stable CF disease and a respiratory exacerbation. Future results from the study will be reported for the molecular differences found that help in predicting an exacerbation.

SAWN offers unique advantages in terms of ease of implementation, non-destructive ionisation conditions, and acquisition and data analysis time in the order of minutes.

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Phosphoproteomics dissection of the cross-talk between MAPK and PI3K-mTOR pathways in different cancer models

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Background
Signalling pathways regulated by MAPK and PI3K-mTOR play key functions in the regulation of cell survival, differentiation, proliferation, metabolism, and motility in response to extracellular stimuli. Both signalling pathways might intersect to regulate each other, co-regulate downstream functions and are deregulated in most cancers. Consequently, pharmacological inhibitors of the MAPK-PI3K network are among the most intensively pursued therapeutic approaches for the treatment of different cancer types.

Methods
Phosphoproteomics methodology was used to analyze three different human cancer cell lines (originating from breast, testis and peripheral blood) treated with MEK and PI3K inhibitors. We carried out label-free LC-MS-based quantitative phosphoproteomics, western blotting and proliferation assays to investigate patterns of cross-talk between the MAPK and PI3K-mTOR pathways.

Results
We found distinct phosphorylation on key sites as a function of treatment with the kinase inhibitors across the cell lines. For example, trametinib, a MEK1/2 inhibitor, increased the phosphorylation of sites specifically in the breast cancer model, but not in myeloid or neuronal cells, suggesting the existence of cell-type specific feedback loops.

Conclusions
We found different patterns of signalling and compensatory cross-talks in human cancer cell lines, thus highlighting cell-specific circuitries that may play roles in how cancer cells respond to kinase inhibitors.

Keywords
Signalling networks, phosphoproteomics, kinase inhibitors, cancer

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Relative Protein Quantification on Model Organisms

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Background
Protein quantification has been a focus of functional proteomics for more than a decade and numerous methods have been developed which allow the system-wide quantitative analysis of proteins and peptides. An efficient and practical bottom-up proteomic workflow is presented which combines isobaric labeling and high-resolution two-dimensional LC-separations with sample concatenation followed by MS analysis. Two studies on established model organisms, E. coli and C. elegans, are presented to display the high proteome coverage and reproducibility of the produced datasets.

Methods
E. coli was cultivated on acetate or glucose as the sole carbon source. C. elegans were grown on bacterial lawns of different compositions including a nematicidal and a non-pathogenic strain of Bacillus thuringiensis. In both studies, proteins were extracted using acid-cleavable detergents, digested by trypsin and labeled with isobaric tags. Peptides were separated using a high/low pH reversed phase 2D-LC separation with an optimized fraction pooling scheme.

Results
The applied two-dimensional reverse-phase liquid chromatography provided high-resolution peptide separation and fraction concatenation mitigates orthogonality issues associated with the use of two reversed-phase separations. The applied Isobaric labeling scheme produced robust and comprehensive relative-quantitative datasets, providing an overview of the system-wide differences in protein abundance under the examined conditions.

Conclusions
The developed labeling and separation scheme allows the combined analysis of multiple biological and technical replicates. The low technical variability of multiplex labeling contributes to the statistical power of the relative quantitative data and both fraction concatenation and multiplexing with isobaric tags significantly cut down on analysis and instrument time.

Keywords
Model organisms; relative quantitative proteomics. high/low pH 2D-LC MS

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Plasma-derived microparticle biomarkers of paracetamol-induced hepatotoxicity

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Paracetamol (APAP) is a world widely prescribed analgesic and antipyretic drug. Incorrectly dosed, it is the most common cause of acute liver failure (ALF) in developed countries. Even used at therapeutic dose (4g/day), up to one third of healthy volunteers as well as hospitalized patients display elevated liver tests after APAP intake in the presence of risk factors such as malnutrition or co-medications. It is therefore crucial to very early detect and diagnose paracetamol-induced hepatotoxicity. Blood microparticles (MPs) are circulating microvesicles whose content is specific to their cell of origin, and reflect pathophysiological states. MPs are used here as potential plasma-derived biomarker holders of APAP-induced hepatotoxicity. MPs were isolated from human whole blood by differential centrifugations, and pelleted from platelet-free plasma after centrifugation at 18.000g for 45 min. Quantitative proteomics strategies (10plex isobaric Tandem Mass Tag, [TMT]) were applied to compare the MP protein content from patients under APAP treatment that encountered liver test elevation, to patients with steady liver test (n=3). Four TMT tags were used to label HepaRG liver cell proteins as references. Data were analyzed with Proteome Discoverer software.
A proteome of blood-circulating MPs was depicted. We identified 819 proteins (1% FDR, at least two distinct peptides), with more than 60% of vesicle-related proteins. Then, the procedure was applied to patients under APAP treatment. Sixty-three significantly differential proteins were observed, and according to biological and pathologic interest, three proteins were selected for further validation. Namely, alpha-1-antitrypsin, alpha-1-antichymotrypsin and platelet glycoprotein 9 regulations were confirmed by western blot.
The present study underlines MPs value for biomarker discovery in the context of APAP-induced hepatotoxicity. Moreover, the proteins found differentially expressed are consistent with the biological context, as they were already described to be associated with hepatotoxicity mechanisms. They therefore represent promising candidates for the discovery of APAP-induced hepatotoxicity biomarkers.

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Ageing influence central carbon metabolism in a tissue specific manner

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Background: Ageing represents the accumulation of physical, psychological changes in living beings over time. Understanding protein dynamics of deregulated molecules will provide useful insight into many metabolic disorders like diabetes, obesity etc. We aimed to identify the deregulated protein and metabolome profile of different mouse organs like liver, brain and muscles in young (4 weeks) and aged (27 weeks) conditions. The outcome of these ongoing activities may provide us the mechanisms or pathways that are altered during ageing.
Methods: We employed iTRAQ method, briefly 100 µg of protein from 27 weeks and 4 weeks old mouse liver, brain and muscle tissues were used. The raw data processing and statistical analysis were done using the MaxQuant and Perseus software respectively. 100 mg of wet tissues were grinded in LN2, lysates were resuspended in phosphate buffer and sonicated. Metabolites were extracted using 100% methanol and metabolite data were acquired using GC-MS after derivatization using MOX and BSTFA.
Results: We identified 55 deregulated molecules with at least 1.5-fold up or down regulation in aged tissues with respect to young one. Out of these, 37, 17 were overexpressed in brain and muscle tissues respectively and only 3 of them showed downregulation. These proteins involve in glycolysis, TCA and central carbon metabolism pathways which we are validating in independent sample sets western blotting. The level of parvalbumin in aged tissues was high in brain as compared to liver and muscle. We also found that D-mannose was overexpressed 4 times in aged muscle tissues with propanoic acid 10 times downregulated. In case of muscle and liver, cholesterol levels were more than 2 times high.
Conclusions: Multiomics data provide interesting insight on the age associated variation at molecular levels. These observations may be explored further in disease associated with ageing.
Keywords: iTRAQ, aged/young, metabolic disorders, proteomics, metabolomics.

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Pathway Analysis of Altered Kinases Reveal Gonadotropin Hormone Signaling in Glioblastoma

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Background: Protein kinase-mediated signaling pathways are known to be altered in glioblastoma multiforme (GBM), one of the most malignant and aggressive forms of primary brain tumors, and are prominent targets in cancer treatment. We wanted to examine enriched pathways revealed after combining all deregulated kinases in GBM observed in highthroughput omics studies followed by pathway analysis.
Methods: In this context, we integrated the expression data at transcriptomic and proteomic levels and screened protein kinases deregulated in GBM, followed by pathway analysis using Ingenuity pathway knowledgebase. We curated the top signalling pathway based on systematic literature survey and the updated generic map was further overlapped to GBM multi-omics data to analyze cross-connectivity to other enriched pathways.
Results: The above analysis revealed a highly significant enrichment of the gonadotropin-releasing hormone (GnRH) signaling pathway that was not deciphered with single omics datasets. The curation of the GnRH pathway with extensive literature analysis brought about a comprehensive annotation of the pathway, which included several additional pathway members that were not previously annotated. We present here an updated generic pathway map of GnRH signaling, show its enrichment in the context of GBM, and plausible cross-connectivity with EGFR, wnt, calcium, and focal adhesion kinase signaling pathways that were earlier shown to be the top deregulated pathways in GBM.
Conclusion: In conclusion, this study demonstrates the promise of multi-omics research and pathway analyses using alternative approaches. Enrichment of GnRH Signalling pathway opens up the new possibility of targeting GnRH signalling pathway in GnRH-receptor expressing GBM, for clinical applications.
Keywords: Glioblastoma multiforme, protein kinases, gonadotropin-releasing hormone

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Platelet proteome reveals novel pathways of platelet activation and platelet-mediated immunoregulation in dengue.

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Dengue is the most prevalent human arbovirus disease worldwide. Dengue virus (DENV) infection causes syndromes varying from self-limiting febrile illness to severe dengue. Although dengue pathophysiology is not completely understood, it is widely accepted that increased inflammation plays important roles in dengue pathogenesis. Platelets are blood cells classically known as effectors of hemostasis which have been increasingly recognized to have major immune and inflammatory activities. Nevertheless, the phenotype and effector functions of platelets in dengue pathogenesis are not completely understood. Here we used quantitative proteomics to investigate the protein content of platelets in clinical samples from patients with dengue compared to platelets from healthy donors. Our assays revealed a set of 252 differentially abundant proteins. In silico analyses associated these proteins with key molecular events including platelet activation and inflammatory responses, and with events not previously attributed to platelets during dengue infection including antigen processing and presentation, proteasome activity, and expression of histones. From these results, we conducted functional assays using samples from a larger cohort of patients and demonstrated evidence for platelet activation indicated by P-selectin (CD62P) translocation and secretion of granule-stored chemokines by platelets. In addition, we found evidence that DENV infection triggers HLA class I synthesis and surface expression by a mechanism depending on functional proteasome activity. Furthermore, we demonstrate that cell-free histone H2A released during dengue infection binds to platelets, increasing platelet activation. These findings are consistent with functional importance of HLA class I, proteasome subunits, and histones that we found exclusively in proteome analysis of platelets in samples from dengue patients. Our study provides the first in-depth characterization of the platelet proteome in dengue, and sheds light on new mechanisms of platelet activation and platelet-mediated immune and inflammatory responses.

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Advanced data-acquisition methods for intact protein analyses by 21 Tesla FT-ICR mass spectrometry

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Top-down LC-MS/MS of complex biological samples is challenging due to the limited time available to acquire data and an inability to continuously optimize data acquisition parameters for each proteoform. Differences in protein size, charge state distribution, and gas-phase structure necessitate use of different fragmentation methods and cumulative ion targets (CIT) to achieve desired sequence coverage in MS/MS spectra.

By direct infusion, multiple “batches” of ETD product ions were accumulated within a multipole storage device prior to detection until maximum sequence coverage was obtained for seven intact protein standards (3-30 kDa). An empirical correlation between optimal CIT and protein MW was derived and incorporated directly within the instrument control software, and applied to on-line LC-MS/MS of an intact protein standard mixture and to GELFrEE fractionated whole cell lysate (WCL). All experiments were performed with a 21 T FT-ICR mass spectrometer.

We demonstrate that the linear operational range in terms of CIT permits acquisition of spectra which enable high sequence coverage without need for time-intensive spectral averaging. An empirical correlation of CIT and protein MW was derived (~1E5 charges per kDa protein) and this relationship incorporated directly into the instrument control software, enabling real-time scaling of the number of ETD fragment ion fills based on observed MW through 50 kDa in online LC-MS/MS experiments. The result is a direct improvement in our ability to perform time-efficient, top-down proteomics. For example, in an analysis of WCL, nearly 800 more spectra were acquired when CIT is varied based on MW than with fixed CITs appropriate for the larger proteins in the sample. Future efforts will focus on modulating the type of fragment ion load to obtain optimal sequence coverage.

Through the culmination of novel data acquisition methods and instrumentation, 21 T FT-ICR stands ready to accelerate top-down proteomics research.

Top-down, FT-ICR, 21 Tesla

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Novel Highly Sensitive, Selective and Reproducible Method for Quantitative Analysis of Redox Cysteine Modifications

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Background
Highly nucleophilic and redox sensitive cysteine (Cys) residues are subject to variety of post-translational modifications (PTMs) such as, S-sulfenylation (SOH), S-nitrosylation (SNO), S-sulfinylation (SO_2H) and mixed disulfide (RS-SR) formation. The non-enzymatic Cys modifications mediated through cellular reactive nitrogen and oxygen species are increasingly identified as important players in redox mediated cell signalling and protection against oxidative damage. The study of these low abundant, labile and dynamic modifications represents a significant challenge and requires highly efficient enrichment.

Methods
For the analysis of the Cys-PTMs we apply multidisciplinary research tools in quantitative proteomics, chemical biology and biochemistry.
We developed a novel chemical proteomics-based enrichment strategy for Cys-PTM analysis, based on the use of the iodoacetamide alkyne for the switch assay and a biotin-azide cleavable linker for the enrichment. Moreover, we systematically evaluated and optimised the performance of the commercial iodoTMT-based assay. The recovery of anti-TMT enrichment was defined using model synthetic peptides.
The novel chemical proteomics-based strategy, in combination with NHS-TMT labelling, was compared to the iodoTMT approach by enrichment of the total Cys from TCEP reduced HeLa cell lysate in n=3 technical replicates (20 µg protein each) followed by LC-MS/MS.

Results
Superior specificity (98% vs. 31%) and sensitivity (7690 vs. 2246 Cys peptides, 115 vs. 37 Cys/µg protein, and 2800 vs. 1320 proteins) were achieved with our novel chemical proteomics-based enrichment in comparison to the iodoTMT approach. Our method showed higher technical reproducibility and reporter ion intensities, resulting in more confident quantification.

Conclusion
By direct comparison we demonstrated that our novel method clearly outperforms the widely-used iodoTMT assay. Moreover our method is superior to existing methods of total Cys studies. The high specificity and sensitivity of the method render it suitable for the quantitative analysis of endogenus cysteine oxidative modifications.

Keywords: Chemical proteomics, cysteine modifications, click chemistry, affinity purification

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In-cell structural analysis of MeCP2 – a structural basis for (P225R) Rett syndrome?

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Background
Methyl-CpG-binding protein 2 (MeCP2) is a DNA-binding protein whose activity depends upon interaction with associated proteins. Mutations in MECP2 can cause Rett syndrome (RTT), a severe neurological disorder that affects around 1 in 10,000 live female births. The P225R mutation lies in isolation, outside the two crucial functional domains of MeCP2 that host the majority of RTT-causing missense mutations. We hypothesise that substitution of proline to arginine in P225R disrupts protein structure.

Methods
HEK293 cells were grown in DMEM depleted medium. Photo-amino acids were added, cells cultivated for 22 h and UV irradiated. Cells were lysed and DNA digested, to release DNA bound MeCP2. Immunoprecipitation of GFP-tagged MeCP2 was carried out using anti-GFP antibody coated beads, which was subsequently processed by SDS-PAGE. Gel bands corresponding to MeCP2 were excised and processed for in-gel tryptic digestion, followed by analysis on a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer. Database search revealed links, which were quantified using Skyline.

Results
We have developed a strategy for the structural analysis of wild type MeCP2, via incorporation of photoactivatable amino acid analogues into human cells followed by cross-linking/mass spectrometry. Introducing a label-free quantitative cross-linking/mass spectrometry (QCLMS) approach allows quantifiable comparison between MeCP2 and mutants.

Conclusions
QCLMS data shows differences between wild type and P225R MeCP2, suggesting gross structural deviations over an area of around 100 residues surrounding the P225R mutation. Our analysis platform appears to hold promise for understanding the structural basis of other proteins within human cells.

Keywords
Cross-linking/mass spectrometry, Rett syndrome, photoactivatable amino acids, quantification

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Efficient proteomic and proteogenomic searches with IdentiPy

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Title: Efficient proteomic and proteogenomic searches with IdentiPy
Background: Search engines are at the core of the bottom-up proteomic workflow and have a key impact on the outcome of the study. Care must be taken to ensure adequate search settings and sequence databases are used, as failure to do so can invalidate the results. Recently, more complicated workflows have been introduced, involving genome- or transcriptome-level information translated into specialized protein sequence databases. These databases are used with regular search engines to identify protein sequence variations predicted for a given sample from genomic or transcriptomic information.
Methods: IdentiPy is implemented in Python using the previously developed Pyteomics library. It has a web user interface, IdentiPy Server (built using the Django framework) or can be run from command line. It has several unique features, such as “auto-tuning” of parameters and built-in identification of sequence variants and point mutations.
Results: In regular proteomic searches, IdentiPy shows search sensitivity similar to the current state of the art (MS-GF+), slightly exceeding it on some data sets and conceding on others, and surpasses other popular search engines. Auto-tuning spares a lot of human time and increases the amount of reliable PSMs.
When a database of variant sequences is available, IdentiPy Server will automatically perform separate filtering of variant peptide identifications.
IdentiPy is also capable of searching for single amino acid substitutions, thus allowing searching for variant peptides without genomic databases. To improve selectivity, IdentiPy requires fragment-ion evidence for the reported mutations.
Conclusions: IdentiPy is a highly efficient, open-source proteomic search engine equipped with a web-based user-friendly GUI. It is capable of identifying variant sequences without genomic databases by searching for single-residue mutations and has built-in support for variant database search.
Keywords: proteomics, search engine, peptide identification, proteogenomics

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A new workflow for deep proteome profiling of the human sperm

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Sperm are highly specialized cells that acquire specific morphological traits during the process of spermatogenesis, which include undergoing meiosis, cell polarization, the formation of a flagellum, and formation of a sperm head which includes highly compacted DNA and an acrosome. The compact structure renders sperm resistant to lysis comparing to most somatic cells. Although normally millions of cells are produced daily, patients with alterations to testicular function may present very few sperm in the ejaculate. The goal of this study was to establish a new protocol for proteome profiling using less than two million sperm.

Following semen liquefaction, samples were centrifuged and subsequently submitted to density gradient to remove contaminating round cells. Isolated sperm were then lysed with SDS and deoxycholate buffer and total protein content was assessed using the modified tryptophan fluorescence method. Proteins were extracted using Trizol and digested using modified eFASP. Tryptic digest were analysed by nanoUPLC-quadrupole-orbitrap instrument operating at data-dependent acquisition method.

While the protein concentration is routinely measured by the BCA or Bradford assays, they do not provide sensitivity to work with a small number of sperm cells. Using tryptophan fluorescence, low protein amounts from 0.5e5 to 2e6 cells were precisely determined. Trizol method coupled to a solubilization buffer with SDS and deoxycholate showed efficient cell lysis and good protein recoveries. The application of eFASP allowed complete removal of detergents and high peptide recoveries resulting in high protein coverage in 90 minute analysis.

Assessing the sperm proteome has been one of the most promising tool to understand specific alterations which contribute to the determination of male infertility. In this study we established a method to obtain high recovery of proteins on research projects with samples from patients with very few sperm in the ejaculate.

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MRM and PRM Assay Development for a Panel of >3,000 Proteins from 20 Mouse Tissues

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Background
Mouse is the most widely used animal model in health-related research, however protein expression studies are challenging due to the lack of rapid and robust tools for quantitation. We are developing multiplexed assays to quantify 3000 proteins across 20 mouse tissues by MRM and PRM mass spectrometry using stable isotope labeled standard (SIS) peptides.

Methods
Untargeted LC/MS/MS for 42 mouse tissues was performed on an Orbitrap Fusion. Peptide and protein identifications were assigned and 2 proteotypic tryptic peptides selected for each target protein. Corresponding 13C/15N-labelled SIS and unlabeled (natural, NAT) peptide pairs were synthesized in-house. Tissues were digested overnight and synthetic peptides spiked into samples before SPE clean-up and analysis on an Agilent 6495 QqQ or ThermoFisher Q-Exactive. Assay LLOQ and linear range was characterized using standard curves and assay variability was calculated from samples prepared and measured on 5 separate days.

Results
Untargeted Orbitrap analysis of >20 mouse tissues identified 12057 proteins and informed the selection of targets. Our synthetic peptide library currently contains 1663 peptides from >800 mouse proteins; peptides for an additional 3000 proteins will be synthesized during this project. Following transition optimization, peptides are screened for interferences in tissue samples. For assay development, standard curves containing a constant amount of NAT as the internal standard and a 32,000-fold range of SIS were generated in sample matrix to determine LLOQ and linear range. Assay variability was evaluated at 2.5x, 50x, and 500x the LLOQ over 5 days, where the total variability must be <20%. To date, MRM assays have been developed for 731 proteins in mouse plasma and 197 proteins in heart tissue. Additional protein assays in other tissues are in progress.

Conclusions
These assays allow rapid characterization of protein expression in various mouse tissues, enabling deeper biological questions to be addressed.

Keywords
Quantitative MRM, Mouse

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Integrated proteo-glyco-genomics identified the potential clinical target of cancer stem cells

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Background

Cancer stem cells (CSC) have been considered responsible for the therapeutic-resistance/recurrence of cancers, and thus proposed as the therapeutic target. To clarify molecular mechanisms and develop clinical targets against CSC, we established CSC-clones from glioblastoma patient's tissues as glioma stem cells(GSCs), having a potential of differentiation/promotion of glioblastoma, and subjected to a unique integrated proteo-glyco-genomics. Using original GSC-iPEACH database, we tried to identify specific glyco-related networks associated to the maintenance/differentiation of GSC.

Methods

We established 9 GSC clones from patient's gliomas having the potential to differentiate into glioblastomas, extracted their Proteins and mRNAs, and subjected to iTRAQ/TMT, DNA array, Rectin and glyco-gene array analyses. All of the data was integrated by iPEACH, and used for GO and knowledge-based network analyses. Biological validations were performed with immunocyto/histochemistry, western-blotting, mouse xenograft analyses.

Results

The data integration and extraction of global proteins, mRNAs and glyco-genes revealed that, during the GSC differentiation, cell adhesion molecules including integrin aV/ECMs and RAS-MAPK/PI3K signalings were significantly up-regulated, meanwhile, SOX2, CD133, and specific proteoglycans/synthetic-enzymes/metabolic pathways were obviously down-regulated. Among them, we focused proteoglycans and their synthetic-enzymes. GSC differentiation was significantly associated with the decrease of CSPG(CS-modified form) and dramatically induced by the CS-degradation enzyme which also induces the up-regulation of ERK-AKT signaling and GFAP without any other agents. Importantly, these differentiation processes were also associated with the interaction of CSPG(CS-unmodified form) and integrin-aV, and suppressed by integrin-inhibitors/CS administrations significantly. Combination treatments of a cancer-drug Temozolomide and these GSC-differentiation inhibitors suppressed glioma progression, increased the chemosensitivities, and led the longer survival of mouse xenograft-models.

Conclusions

Functional integrated proteo-glyco-genomics for the first time demonstrates that the GSC induces the specific proteoglycans to regulate GSC stemness/differentiation via the integlin signalings which may be a clinical target against malignant gliomas.

Keywords

Integrated proteo-glyco-genomics, Glioma, Cancer Stem Cells, proteoglycans

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Understanding the Effect of Deisotoping MS/MS Spectra on Protein Identification

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Background:
Robust protein identification by tandem mass spectrometry requires the detection of the monoisotopic peak of both the peptides and their fragments. Despite this, identifying the monoisotopic peaks from fragmentation spectra can be difficult due to the existence of heavy isotopic peaks. Deisotoping algorithms have been used to eliminate isotopic peaks and thus improve detection of the monoisotopic peak. However, understanding how the removal of isotopic peaks improves protein identification and the extent of the improvement remains unclear, which has hindered the optimization of this process.

Methods:
To address this, we developed Easy-Dei-MS, a tool to identify monoisotopic peaks and remove their isotopic peaks from fragmentation spectra. This is done by comparing the mass and/or relative intensity of each peak to every other higher mass peak. To better understand how deisotoping improves protein identification, we used Easy-Dei-MS to remove isotopic peaks in raw tandem mass spectra from yeast and human and performed large-scale MS/MS ion searches. MS/MS data from searches with raw and deisotoped fragmentation spectra were then compared to assess how deisotoping improves protein identification.

Results:
In both yeast and human, we found that the Mascot ion scores for PSMs change depending on the deisotoping method applied. On average, removing isotopic peaks by comparing the mass of peaks decreased Mascot ion scores by 10, whereas comparing the mass and relative intensity of peaks increased Mascot ion scores by 3. When applied on a large-scale, deisotoping resulted in the identification of an additional 2,600 PSMs and 30 proteins.

Conclusions:
We highlight the advantages of deisotoping fragmentation spectra to increase the number of proteins that are identified in database searches, and demonstrate the importance of comparing the mass and relative intensity of peaks in deisotoping algorithms.

Keywords:
Mass spectrometry, data analysis, proteomics

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Fast and accurate protein false discovery rates on human proteome study scale with Percolator 3.0

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Percolator is a popular tool to assign reliable statistics, such as q-values and posterior error probabilities, to peptides and peptide spectrum matches (PSMs) using search results from mass spectrometry-based proteomics experiments. Percolator's processing speed has been sufficient for typical data sets with hundreds of thousands of PSMs. With our new scalable approach, we can now also analyze millions of PSMs in a matter of minutes on a commodity computer. Furthermore, with the increasing awareness for the need for reliable statistics on the protein level, we compared several easy-to-understand protein inference methods and implemented the best method in the Percolator package. We used Percolator 3.0 to analyze the data from a recent study of the draft human proteome containing 20 million spectra (PM:24870542).

Searching all spectra of the draft human proteome study resulted in approximately 73 million target+decoy PSMs and 300,000 unique target peptides. Using subsets of even just 100,000 PSMs (0.1%) for SVM training did not reduce the number of identified peptides and generally even slightly increased this number. The standard deviation of the randomized runs for a fixed subset size did increase when taking increasingly smaller subsets, but this effect was limited. By using a subset of 500,000 PSMs to train the SVM, Percolator’s runtime was reduced from several hours to under 10 minutes.

We compared several straightforward protein inference methods: Fisher’s method for p-value combination, the picked target-decoy strategy, and the product of peptide-level posterior error probabilities (PEPs). For the draft human proteome set, at 1% FDR, the picked target-decoy strategy identified 12,300 proteins, multiplication of PEPs 11,600 and Fisher’s method only 8,800. From these results we concluded that the picked target-decoy strategy was the superior alternative and implemented this in the newest Percolator package.

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Up to ~10,000x faster DIA Processing using DeepSearch: Results During Your Coffee Break

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Currently, DIA informatics processing times are astronomical. For even a moderately sized project of 100 samples, total true cost of informatics processing time (including DDA informatics processing to generate spectral libraries for the DIA algorithms) time is literally measured in weeks on current MS vendor solutions.

Here, we've developed DeepSearch: a fully pipelined and automated solution that not only identifes and reproducibly quantifies far more low abundant peptides than existing state-of-the-art solutions, but it also does so at up to ~10,000x faster than current solutions, achieving results for ~100 samples in approximately the time it takes for the average researcher to finish her coffee.

At these speeds and by breaking the current informatics bottleneck barrier, worlds of possibilities open up to the DIA MS research practictioner, and we describe and elucidate different use-case scenarios from half-dozen labs from different backgrounds (militarized government; big pharma, big hospital facility; two core labs; and several academic research labs).

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A protocol for finding missing proteins

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In the quest to uncover the entire human proteome, finding "missing proteins" remains the Holy Grail of scientists. In order to capture existing information, in addition to high-stringency MS data, we have launched the MissingProteinPedia (MPP; missingproteins.org), as an integrative biological database. While MPP incorporates automated data collection, novel tools for functional annotation and collated publications, there is an urgent need to identify a protocol for evaluating MPP data, to facilitate missing protein annotation jamborees. We will present and discuss several options towards arriving at a protocol for evaluating "extraordinary evidence" for missing proteins and welcome inputs from the HuPO community to arrive at a workable and efficient approach.

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Functional determination of a single methylation site in the human proteome using CRISPR/Cas9 and MS/MS

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Background

Lysine methylation is now known to be widespread in the human proteome, however the enzymes that catalyse its addition remain largely undefined. As a consequence, it has been difficult to investigate the functional role of any single methylation site. Here we demonstrate how this can be achieved, through use of CRISPR/Cas9 knockouts and detailed proteomic analysis, for the discovery of a new protein methyltransferase and the characterisation of function.

Methods

We aimed to discover the methyltransferase responsible for methylation of lysine 165 of eukaryotic translation elongation factor 1A (eEF1A). The CRISPR/Cas9 system was used to knock out putative protein methyltransferases METTL21B and METTL23 in K562 cells. The known eEF1A methyltransferase EEF1AKMT1 was also knocked out as a control. Targeted MS/MS was used to assay for loss of methylation and SILAC and MS/MS analysis was subsequently used to characterise the effect of knockdowns on the K562 proteome.

Results

Knockout of METTL21B abolished the methylation of eukaryotic translation elongation factor 1A, at lysine 165. In vitro assays, whereby recombinant METTL21B was incubated with unmodified elongation factor 1A, confirmed the methylation activity of the enzyme at this site. Whole proteome analysis of knockouts revealed a striking result: the cell upregulates the biogenesis of the large ribosomal subunit on loss of K165 methylation on elongation factor 1A. Given that the elongation factor interacts with the ribosome, this suggests that this methylation event functions to improve the rate and/or efficiency of the translational process.

Conclusions

This is the first study to discover human methyltransferases by CRISPR/Cas9 and proteomic techniques, and the first characterisation of the human proteome on loss of a single methylation site. More generally, our results demonstrate the power of such approaches for the functional characterisation of single post-translational modifications.

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PROTEOME-WIDE STUDY OF CEREBROSPINAL FLUID BIOMARKERS FOR BIPOLAR DISORDER AND OTHER NEUROPSYCHIATRIC DISORDERS

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Background
There is a great need for expanding our knowledge on molecular mechanisms that underlie bipolar disorder (BD) and other neuropsychiatric disorders. Cerebrospinal fluid (CSF) biomarkers may aid in the diagnostic assessment, elucidate pathogenic pathways of the disease, be used for monitoring and prediction of effects of therapies and provide prognostic assessments.

Methods
For biomarker discovery, we have developed an exploratory proteome-wide approach for identification and quantification of CSF proteins and peptides. Our approach is based on a highly sensitive liquid chromatography-mass spectrometry analysis with Tandem mass tag (TMT®) labeling for multiplexed quantitative analysis.

Results
In a pilot study of 15 BD type 1 patients and 15 age- and sex-matched controls, 676 proteins (4892 peptides) were quantified. Of these, 36 proteins had significantly ($p<0.05$, not adjusted for multiple testing) different CSF protein levels in the BD type 1 patients compared to controls. The majority of these proteins were central nervous system-specific as synaptic proteins, neuroinflammation molecules, proteins involved in calcium channel functionality and extracellular matrix proteoglycans. A subset of the proteins has previously been implicated in BD on genetic level (Neurocan, Cav1.2 calcium channel subunit) while several proteins are novel findings (e.g. Cadherin 13, Neurexin 1, Complement factors, Amyloid precursor protein, Neuronal pentraxin 2). The results are now validated in CSF of two larger independent BD cohorts (N=122 and 226). With a pre-fractionation step during sample preparation, we now can quantify approximately 1300 proteins in one single CSF sample.
We are also working in the field of schizophrenia, autism spectrum disorder and ADHD with collection of CSF samples and large-scale analysis of potential biomarkers.

Conclusions
This is the first proteome-wide CSF study of BD, demonstrating the feasibility for identifying potential biomarkers for BD and for unraveling molecular pathophysiological mechanisms.

Keywords
Bipolar disorder, Biomarker, Cerebrospinal fluid, Quantitative proteomics, Tandem mass tag

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The analysis and identification of STAT3 interactions and modifications in the mitochondria of cancer cells.

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Introduction
STAT3 belongs to the family of Signal Transducer and Activator of Transcription proteins that are highly conserved in evolution and crucial to life. Traditionally STAT3 is known for its role in the nucleus, targeted through phosphorylation at Y705, where it controls gene transcription and regulates key biological processes. Deregulation of this process leads to many human diseases, including a host of human cancers. More recently, STAT3 has been shown to play a mitochondrial role, but its function, import method and specific mitochondrial interactions remain a mystery. It has however shown to be critical in the development of mitochondrial oncogene tumour growth and control, making it a potential drug target for cancer research.

Methods
Tagged STAT3 was stably over-expressed in 2 independent cancer cell lines. STAT3 and its binding partners were isolated from subcellular compartments by immunoprecipitation. These were then subjected to digest and purification, peptides analysed using an ABSCIEX 5600 NanoLC/triple-tof mass spectrometer. We repeated our IP/MS approach on WT cells lacking tagged STAT3 as controls for non-specific purifications. For data analysis, we used different search algorithms (PEAKS, Protein Pilot, and MASCOT) to address specific experimental questions.

Results
After eliminating non-specific interactions and comparing putative interacting proteins between subcellular compartments from within the same cell, we identified a number mitochondrial STAT3 binding proteins that are key in mitochondrial transcription/translation control. These proteins have since been confirmed by in-vivo IP and have given us insights into the role STAT3. We were able to obtain 96% peptide coverage, allowing us to also identify new post-translational modifications within STAT3 that have proven important for mitochondrial localisation and function.

Conclusions
We have identified a key role of STAT3 in the mitochondria and the target sequence, which will have wide implications for future medical and cancer research.

Keywords
STAT3, Mitochondria, Cancer

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Zwitterionically Functionalized Soluble Nanopolymers Used For Efficient Glycopeptides Enrichment

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Highly selective enrichment is essential for mass spectrometry analysis of glycoproteome study. Poly (amidoamine) dendrimer (PAMAM) which was adopted for the synthesis of zwitterionically functionalized (ZICF) materials has achieved satisfactory results for N-glycopeptide enrichment. It has the minimum detectable concentration of femtomolar level and high recovery rate of over 90.01%, and can e ciently enrich glycopeptides from complex biological samples even for merely 0.1 µL human serum. After the optimization of experiment condition, Poly (amidoamine) dendrimer (PAMAM) also can effectively enrich O-glycopeptide and has already gotten effective results of standard proteins. The remarkable glycopeptides enrichment capacity of ZICF-PAMAM highlights the potential application in in-depth glycoproteome research, which may open up new opportunities for the development of glycoproteomics.

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Proteome and Transcriptome Analyses of Phosphorylation Site–Specific Mutants of BRG1, a Chromatin Remodeling Component

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Background
The SWI/SNF chromatin remodeling complex contains many tumor suppressors (e.g., ARID1A and BRG1) that are frequently mutated or downregulated in various cancers. High mutation rates in SWI/SNF genes (~75%) were observed in ovarian clear cell carcinoma (OCCC), a malignant subtype of ovarian cancers. Previously, we reported that the phosphorylation level of BRG1 was significantly reduced in some ARID1A-positive OCCC cell lines (Kimura et al., 2014), suggesting the involvement of both ARID1A and phospho-BRG1 in malignancy. To reveal the role of BRG1 phosphorylation, we analyzed the transcriptome, proteome, and phenotypes of cell lines with or without mutations in the phosphorylation site of BRG1.
Methods
Plasmids containing wild-type, phosphorylation-mimic, or phosphorylation-deleted alleles of BRG1 were transfected to BRG1-negative cells. Stable clones were picked by selection with G418. Proteins extracted from these clones were digested, and phosphopeptides were enriched using TiO_2 resin. Peptides were analyzed by LC-MS/MS using Q Exactive (Thermo Fisher Scientific) and subjected to quantification/multivariate statistical analysis by Progenesis QI (Waters). RNA sequencing was performed by analyzing cDNA libraries on an Illumina HiSeq 1500 (Agilent Technologies). Proliferation and invasion/migration were analyzed by MTS and scratch assays, respectively.
Results
Proteome and phosphoproteome analyses revealed the significant up- or downregulation of 308 proteins (including 47 phosphoproteins) in phosphorylation-mimic or phosphorylation-deleted mutants ($p<0.01$). Transcriptome analysis revealed similar quantitative differences in some genes. With regard to phenotypic differences, phosphorylation-mimic cells proliferated more slowly than wild-type or phosphorylation-deleted cells. In addition, the invasive and migratory abilities of phosphorylation-deleted cells were elevated in comparison with wild-type cells, whereas those of phosphorylation-mimic cells were significantly reduced ($p<0.05$).
Conclusions
Our proteomic and transcriptomic analyses revealed many genes and proteins that were up/downregulated in phosphorylation site–specific mutants of BRG1, and are thus potentially involved in the malignant features of cancers.
Keywords
phosphorylation, BRG1, cancer, malignancy

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Quantitative map of beta-lactone induced virulence regulation

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Background
Beta-lactones are selective inhibitors of ClpP, an important protease complex in bacteria and eukaryotic cells. Usually, bacteria attack their hosts with a diverse set of virulence factors, mainly secreted proteins such as alpha-toxin. beta-lactones, however, attenuate virulence of drug-resistant Staphylococcus aureus. But a link between beta-lactone inhibition and molecular virulence mechanisms has been lacking so far.

Methods
We performed a chemical-proteomic approach to elucidate anti-virulence pathways in S. aureus. In a first step, we used chemical probes of beta-lactones to confirm ClpP as an in situ target with a combination of pull down and mass spectrometry (affinity-based protein profiling). Then, we chemically knocked out ClpP and analyzed resulting global expression changes on the proteome level. Finally, we built up a virulence regulation map by putting literature data of functional protein networks together with our quantitative proteomics data.

Results
ClpP is the predominant target of beta-lactones in S. aureus cells. In-depth mechanistic insight was provided by a full proteomic comparison between lactone treated and untreated S. aureus cells. This revealed increased Rot levels. It is together with RNAIII a major controller of virulence. Furthermore, we detected a down-regulation of alpha-toxin. With the help of the quantitative virulence regulation network, we finally highlighted the impact of ClpP inhibition in a systems biology context.

Conclusions
We provide a link between beta-lactone, ClpP and virulence by the observed upregulation of Rot, a major repressor protein of S. aureus pathogenesis. Additionally, a closer inspection of the RNAIII/Rot surrounding network reveals interesting systems biology insights. Therein, RNAIII is a key regulatory element whose abundance is strictly controlled over time. It acts mainly via Rot and we hypothesize that its protein levels are dependent on ClpP activity, likely via ClpX chaperone levels.

Keywords
S. aureus ClpP; antivirulence; activity-based protein profiling; chemical knockout

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The Encyclopedia of Proteome Dynamics – A big data ecosystem for (prote)omics

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Background: There is a continuous growth in datasets of both raw MS files and processed peptide and protein identifications. MS-based proteomics technology is also used increasingly to measure additional protein properties affecting cellular function and disease mechanisms, including PTMs, protein-protein interactions and subcellular and tissue distributions. Consequently, biologists and clinicians need innovative tools to conveniently analyse and visualise such large, complex proteomics data and to integrate proteomics data with genomics information and other related large-scale data sets
Methods: The EPD was developed as polyglot persistent ecosystem using both Cassandra and Neo4j as database storage engines. It is also a webapp that was implemented on Django with nginx. It integrates proteomics studies from the Lamond Laboratory and close collaborators along with public databases like CORUM and GO. It is publicly accessible at https://peptracker.com/epd/
Results: The EPD integrates many proteomics datasets from human, including data from multiple cell types, as well as data from the model organisms. It currently stores data for >142 million psm (PSM), > 790,000 distinct peptide sequences and >30,000 proteins.
On the analysis and visualisation aspect, it hosts >100 different interactive visualisations available, ranging from volcano plots, heatmaps, bubble plots, bar plots, box plots, scatter plots to parallel coordinate plots.
Conclusions: The EPD provides a user-friendly, open access big data solution for the integration, visualisation and analysis of large, complex proteomics and related omics data sets. It is built with scalability and resilience as key goals and specifically created to facilitate interactive data exploration and analysis using either desktop, or mobile devices. The EPD data model and interactive analytics platform can be adapted and applied also to many different types of biological and clinical data, providing a paradigm for open access data sharing and integration
Keywords: proteomics, big data, visualisation, omics, analytics

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A novel FAIMS interface extend the sensitivity and depth of proteomic analyses

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Despite remarkable advances in MS sensitivity and resolution, depth of proteomic analyses is often limited by the identification and quantitation of low abundance peptides. In this context, we have developed a new high field asymmetric waveform ion mobility spectrometry (FAIMS) interface that improves ion sampling and peak capacity compare to the previous FAIMS interface. Infusion of BSA with FAIMS provided a 3-fold increase in peptide detection with more than 10-fold improvement in signal to noise ratio compared to conventional electrospray. Replicate LC-MS/MS analyses (n=3) of tryptic digests of HEK293 cells performed with and without FAIMS enabled the identification of 12783 peptides (3017 proteins) and 6253 peptides (2197 proteins), respectively. Improved ion detection was observed for low abundance peptides and extended the limit of detection by one order of magnitude. The reduced precursor ion co-selection observed using FAIMS resulted in lower peptide identification score threshold for a false discovery rate of 1% (score of 13 and 15.6 with and without FAIMS, respectively). The reduction in chimeric MS/MS spectra was advantageously exploited in quantitative proteomics using isobaric labeling. We compared TMT-based quantitation for LC-MS/MS analyses performed using synchronous precursor selection (SPS) and LC-FAIMS-MS/MS to profile temporal changes in protein abundance of HEK293 cells following heat shock for up to 10h. FAIMS provided a three-fold increase in the number of quantifiable peptides compared to non-FAIMS experiments (7754 peptides from 3800 proteins for FAIMS vs. 2038 peptides from 1583 proteins with SPS). LC-FAIMS-MS/MS analyses enabled the identification of 17 heat shock proteins (HSPs) showing a progressive increase in abundance over time compared to only 7 HSPs when the same analyses were performed using LC-MS/MS with SPS. Altogether, enhancement in ion transmission and duty cycle of the new FAIMS interface extended the depth and comprehensiveness of proteomic analyses and improved the precision of quantitative measurements.

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Identification of candidate diagnostic serum biomarkers for Kawasaki disease using proteomic analysis

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Background
Kawasaki disease (KD) is a systemic vasculitis and childhood febrile disease that can lead to cardiovascular complications. Although early diagnosis of KD is crucial for preventing complications, the diagnosis of KD depends on its clinical features, and thus it is often difficult to make definitive diagnosis. Diagnostic methods based on biomarkers are necessary to assist diagnosis of the disease and aid selection of the best treatment. Therefore, we tried to discovery diagnostic serum biomarkers for KD.
Methods
We explored serum KD-related proteins, which differentially expressed during the acute and recovery phases of two patients, using MS-based proteomic analysis for the identification of differentially expressed proteins. We subsequently validated KD-related proteins using western blot analysis and microarray ELISAs.
Results
By using MS analysis, we identified 65,514 peptides (1,813 proteins) for relative quantitative analysis, and found that four proteins were either increased or decreased during the acute phase of KD compared to its recovery phase. In order to evaluate the usefulness of these proteins as biomarkers, we analyzed a total of 270 clinical samples by using ELISAs. Over the course of this experiment, we determined that the expression level of these proteins changes specifically in the acute phase of KD, rather than the recovery phase of KD or other febrile illness.
Conclusions
Environmental factors and genetic backgrounds may affect the expression of proteome, indicating that disease-specific factors are difficult to identify by comparison between different individuals. To screen for KD-related proteins, we used paired sera from KD patients during the acute and recovery phases in this study. In this manner, we successfully identified KD-related proteins for use in effective monitoring of KD phase. We concluded that these proteins are potentially useful for establishing a new method to facilitate early and uniform KD diagnosis.
Keywords
Mass spectrometry, Kawasaki disease, Biomarker

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Analysis of mitochondrial protein interactome by in-organello chemical crosslinking and mass spectrometry

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Background
Chemical crosslinking combined with mass spectrometry is a structural proteomics approach that provides information on both the structure and interactions of proteins. Crosslinking, when applied in vivo or in-organello, permits the analysis of interaction networks on an entire cell or organelle proteome.
We used CBDPS and in-organello crosslinking for the proteome-wide analysis of protein-protein interactions in yeast mitochondria.
Methods
Purified active yeast mitochondria were crosslinked with CBDPS under physiological conditions. After digestion of proteins with trypsin, crosslinked peptides were enriched with SCX, and affinity-purified with avidin. Peptides were analysed by LC-MS/MS (Orbitrap Fusion Lumos Tribrid) using data-dependent acquisition, CID (NCE=35%) at 120K (MS1) and 60K (MS2) mass resolution. Peptides were searched using Kojak software. We have improved the identification of the inter-protein crosslinks by incorporating information on cleavage products in the data analysis. Peptides were identified at a <5% FDR.
Results
Fractionation of peptides with SCX resulted in an overall enrichment of crosslinker-modified peptides, and increase in the relative amount of inter-peptide crosslinks. The affinity purification led to an additional several-fold increase in the number of identified crosslinks. More than 10,000 unique crosslinker-modified peptides - including >1000 inter-peptide crosslinks - were identified. The inter-peptide crosslinks correspond to ca. 24% of all mitochondrial proteins, including matrix and inner membrane proteins. We have detected both known (i.e., present in databases) and new protein interactions.
We have identified inter- and intra-protein crosslinks that can be mapped onto the protein/protein complexes crystal structures with Cα-Cα distances of <32.5Å. Also crosslinks were found for portions of proteins for which no crystal structures are available, thus providing additional structural information that can be used for modelling.
Conclusions
We have developed and implemented a workflow for investigating in-vivo protein-protein networks based on an isotopically coded CID-cleavable affinity-tagged crosslinker CBDPS.
Keywords
In-vivo crosslinking, protein interactions, CBDPS, mitochondria.

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Application of High-throughput Serum Proteomic Strategy Using DIA-MS

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Background

Research aimed at identifying clinical biomarkers using quantitative proteomics approaches often focuses on serum. However, due to the wide dynamic range of protein concentrations, the myriad types of proteins in serum, and differences among individuals, serum samples represent the most complex human proteome. Serum protein fractionation helps to reduce complexity, but decreases the throughput of proteome analysis. To achieve comprehensive and rapid analysis, the data-independent acquisition (DIA) approach is effective. The DIA approach was designed to achieve quantification that is both efficient and reproducible by acquiring MS/MS data of all ions. We applied the DIA approach to serum proteome analysis.

Methods

Highly abundant proteins were depleted from serum using a MARS-14 column. The resultant depleted samples were digested and desalted for analysis by QExactive MS. A spectral library was constructed by data-dependent acquisition (DDA) MS analysis, and differentially expressed proteins were identified by DIA using Biognosis HRM Calibration kit with the Spectronaut software. Using this approach, we identified proteins related to Kawasaki disease (KD).

Results

We constructed a spectral library containing 1,473 serum proteins. On average, ~700 proteins were identified in DIA analysis using the spectral library. Previously, it took 3 days to analyze one sample by DDA (shotgun) analysis to detect differentially expressed serum proteins, but DIA analysis took only 3 hours. Using DIA analysis, we efficiently detected KD-related proteins in serum.

Conclusion

DIA analysis was useful for rapidly detecting differentially expressed serum proteins. Using DIA analysis, we detected efficiently KD-related proteins.

Keywords

Serum, DIA, mass spectrometry

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Endoglycosidase-assisted high confident identification of intact glycopeptides

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Detailed characterization of glycoprotein structures requires determining both the sites of glycosylation as well as the glycan structures associated with each site. In this work, we developed an analytical strategy for characterization of intact N-glycopeptides in complex proteome samples. In the first step, tryptic glycopeptides were enriched using ZIC-HILIC. Secondly, a portion of the glycopeptides was treated with endoglycosidase H (Endo H) to remove high mannose (Man) and hybrid N-linked glycans. Thirdly, a fraction of the Endo H-treated glycopeptides was further subjected to PNGase F treatment in 18O water to remove the remaining complex glycans. The intact glycopeptides and deglycosylated peptides were analyzed by nano-RPLC-MS/MS, and the glycan structures and the peptide sequences were identified by using the Byonic or pFind tools. Sequential digestion by endoglycosidase provided candidate glycosites information and indication of the glycoforms on each glycopeptides, thus helping to confine the database search space and improve the confidence regarding intact glycopeptide identification. We demonstrated the effectiveness of this approach using RNase B and IgG and applied this sequential digestion strategy for the identification of glycopeptides from the HepG2 cell line. We identified 4514 intact glycopeptides coming from 947 glycosites and 1011 unique peptide sequences from HepG2 cells. The intensity of different glycoforms at a specific glycosite was obtained to reach the occupancy ratios of site-specific glycoforms. These results indicate that our method can be used for characterizing site-specific protein glycosylation in complex samples.

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Towards an understanding of neoantigen presentation by Human Leukocyte Antigen (HLA) class I

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Background
Mutations in the cancer genome can cause tumours to express neoantigens (mutated peptides) in the context of Human Leucocyte Antigen (HLA) for recognition by T cells. Neoantigens are attractive candidates for immunotherapy due to their cancer-specificity and their ability to elicit immune responses. A key step in the development of neoantigen immunotherapy is predicting which mutated proteins are processed into peptides by the proteasome and presented by HLA molecules. To better understand the underlying features of HLA presentation of neoantigens, we applied a proteogenomics approach to identify mutated peptides from passenger mutations in clonal cell lines.
Methods
HLA class I complexes were isolated from cancer cell lines by affinity purification. HLA-bound peptides were released by acid elution and fractionated by reversed phase liquid chromatography. Individual fractions were analysed by LC-MS/MS using the Orbitrap Fusion and the ABSciex 6600 TripleTOF. MS/MS spectra, acquired in high resolution and with high mass accuracy, were searched against the human proteome using PEAKS software and filtered to 5% false discovery rate. Cell-line specific mutations were extracted from the TRON cell line portal and included in the human proteome database.
Results
Our data set includes 81 neoantigens from 31 cell lines, with a median number of 1 neoantigen per cell line. A clear trend was found between the mutational load and the number of identified neoantigens, which was confirmed by in silico modelling. Neoantigens were located in “hot spots” of HLA presentation and the mutation itself had little effect on the predicted HLA binding affinity. Implementation of these characteristics into a bioinformatics approach allowed for the prioritisation of candidate neoantigens without relying on prediction algorithms or direct identification by mass spectrometry.
Conclusion
Understanding HLA class I presentation of neoantigens enabled the development of a novel bioinformatics workflow for the prioritisation of candidate neoantigens.

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Analysis of proteomic variation in the human population: A comprehensive iPS Cell Proteome

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Human induced pluripotent stem (iPS) cells generated from adult tissues provide unprecedented opportunities for investigating disease mechanisms and understanding the phenotypic consequences of genetic variation within the normal population.

HipSci is a UK stem cell initiative that has created iPS cell lines from both healthy donors and disease cohorts as a resource for the research community (www.hipsci.org). The cell lines are characterized at the level of proteomic, genomic, RNAseq and phenotypic data, thus providing a unique opportunity to study the impact of genetic variation in the human population at both the protein and gene expression levels. An overview of the IPSC production, QC pipeline and initial genetics data were published recently (Kilpinen et al., Nature 2017).

We describe here the extension of the study referenced above to the proteome level. We have carried out proteomic analysis on over 200 human iPS cell lines, established by the HipSci consortium, using TMT-based quantitative strategy. Peptides extracted from ten different cell lines were labeled using TMT10plex reagents and fractionated prior to LC-MS/MS analysis on a Fusion Orbitrap using SPS-MS3 for more accurate quantification.

We have quantified protein expression levels to the depth of ~ 9000 proteins per cell line and > 11,000 in all cell lines combined. Parallel data are available for RNA expression levels for the same cell lines.

We will present an overview of the HIPSCI analysis, including genome-wide maps of common genetic variation in humans that are associated with expression changes at the RNA and protein level. Comparative analysis of these molecular layers has provided a detailed description of natural variation in the human proteome.

All the HipSci proteomics data will be shared openly via our user-friendly, online searchable web-based interface, the Encyclopedia of Proteome Dynamics (www.peptracker.com/epd) and the associated raw files will be publicly available via Pride/Proteome exchange.

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Multi-protease Strategy Identifies More Membrane-associated Missing Proteins in Human Testis Tissue

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BACKGROUND
Although five-year missing proteins (MPs) study have been performed, the MPs searching is still one of mission of Chromosome-Centric Human Proteome Project (C-HPP) in this year. Following the step of C-HPP, we have focused on the testis-enriched MPs by various strategies since 2015. Based on the theoretical analysis of MPs (2017.02, neXtProt) by multi-protease digestion, we found non-conventional proteases could improve the peptide diversity and sequence coverage compared with trypsin.
METHODS
In this study, multi-protease strategy was used for searching more MPs in the same human testis tissues. The extracted total cell lysate were resolved by a 10% SDS-PAGE followed by digestion with trypsin, lys C, Glu C and lysargiNase, respectively. The sliced peptides was separated by high resolution LC-MS/MS system (Q Exactive HF).
RESULTS
Total 7838 protein groups were identified, of which 30 protein groups belong to MPs. Among them, 22 proteins belonging to 8 groups were confirmed as MPs after spectrum quality check, isobaric and single amino acid variants filtering and verification with synthesized peptide. Among these confirmed MPs, 77% MPs were testis-specific and 80% MPs were membrane-associated proteins. Two large MP families were identified in this study, which heavily correlated with multiple types of cancers.
CONCLUSIONS
A multi-protease strategy was applied for testis-enriched MPs with human testis samples. Totally, 30 were saved as MPs in the neXtProt MPs list (2017-2), including 25 MPs from LysargiNase, 21 from Lys-C and 12 from Glu-C digesting datasets. After verification, 22 MPs belonging to 8 groups survived in the final list. These MPs had been proved to have high mRNA level in testis tissues. The multi-protease strategy used in this study may guide further study in other tissues for MPs in the near future.
KEYWORDS
Chromosome-Centric Human Proteome Project, testis, missing proteins, multi-protease

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Revolutionary Proteome Profiling and Quantitation without Compromising Speed, Sensitivity, and Selectivity

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For large cohort studies in translational proteomics research, data independent acquisition (DIA) methods are used to provide a global view of protein abundance changes among the samples. Here, a novel high resolution MS1 based quantitation DIA (HRMS1) method was developed on the new Thermo Scientific™ Q Exactive™ HF-X mass spectrometer to optimize usage of scan speed, ion injection time (sensitivity), and high resolution (selectivity). Conventional DIA quantitation is based on the intensities of fragments in MS2 scans. Instead of quantifying the peptides on MS2 intensitities, MS1 scans offer potentially higher sensitivity since the peptide precursor is not fragmented into multiple fragments. High resolution detection removes interferences from analytes of interest, achieving accurate and precise quantitation.

The samples consisted of three separate proteomes (HeLa, Yeast, and E. coli). For high-throughput analysis, an UltiMate 3000 RSLC system equipped with capillary flow configuration, coupled to Q Exactive™ HF and Q Exactive™ HF-X systems, was used to separate tryptic peptides from three proteomes with 1-hour run time. Different MS1 scan resolution settings s were evaluated to demonstrate the effect of resolution quantitation.

The Q Exactive HF-X system enables collection of a higher number of ions per time can be collected on. The HRMS1 method uses MS2 scans only for identification, and accordingly the sampling rate of MS2 per precursor is kept as low as possible, while the number of MS1 scans is kept constant across the LC peak for precise quantitation. Different resolution settings on the MS1 scans were evaluated. With higher resolution, ~ 10% more peptide precursors are identified with 1% FDR, and ~ 20% more peptide precursors are quantified with CV% < 20%. This result shows that high resolution is the key to achieve accurate and precision quantitation by removing interferences.

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Extraction and analysis of Aβ fractions of the peptidome from human blood components

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Blood is an attractive target for biomarker monitoring of socially significant diseases since it contains a wide dynamic range of proteins and peptides, and can provide important information about the processes occurring within the organism. Alzheimer's disease is a fatal neurodegenerative disease of the elderly, characterized by progressive impairment of various brain functions due to severe accumulation of aggregated amyloid-beta peptides – normal components of blood and CSF. Changes in the ratio of Aβ40 and 42 in CSF are a biomarker used for diagnostics, but CSF collection is a very traumatic procedure and blood analysis would be much more preferable. However, Aβ concentration in blood is quite low and the peptides tend to stick to numerous other media components, complicating extraction, but potentially providing other possible biomarkers of this fatal disease. Also Aβ may be synthesized in tissues other than the brain, as well as absorbed and accumulated in blood components, and thus its levels might not be indicative of the changes ongoing in the brain. Thus an efficient method of peptidome extraction and complex analysis of Aβ fraction composition and origin in different blood components is of great interest.
A variety of extraction methods including different combinations of solvents, differential solubilization, protein precipitation, gel-filtration, ultrafiltration, and several types of solid phase extraction was screened for the highest yield and diversity of the extracted peptides. Also several amyloid-targeted enrichment procedures were tested. As a result a special method using two-step immunoprecipitation was especially designed to isolate, purify and concentrate amyloid-beta from 15mL of freshly collected whole blood so as to analyze its composition. Analysis was carried out using gel-electrophoresis and LC MS/MS. Internal APP and Ab fragments were extracted and conclusions on their origin of synthesis could be made. Also several post-translational modifications could be monitored using the developed method.

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Quantitative Interactome and Phosphoproteome Analysis Identifies Novel Signaling Components of Parkinson's Disease-Associated Kinase PINK1

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Background
Mutations in PTEN-induced putative kinase 1 (PINK1) and Parkin are causative of autosomal recessive familial Parkinson's disease. We have previously reported that PINK1 is activated by autophosphorylation on depolarized mitochondria and phosphorylates both Parkin and ubiquitin to stimulate Parkin E3 activity. Activated Parkin and phosphorylated ubiquitin chains are shown to interact and accumulate on depolarized mitochondria, which are then removed by autophagy. However, the existence of additional components of PINK1 signaling has been suggested and therefore verified by combining various proteomic approaches.

Methods
PINK1 complex was immunoprecipitated from lysates of HeLa cells stably expressing Flag-tagged PINK1 and subjected to on-bead digestion followed by LC-MS/MS analysis. Identified proteins were quantified by the label-free method. To globally identify downstream targets of PINK1, PINK1-expressing and parental HeLa cells were treated with or without mitochondrial uncouplers. Extracted proteins were digested, and peptides were labeled with TMT10plex reagents. Labeled peptides were mixed and subjected to Fe-NTA-based phosphopeptide enrichment and high pH reversed phase fractionation. Each fraction was analyzed by LC-MS/MS.

Results
We identified known components of the PINK1 complex including Cdc37, Tom22, Tom40, and TIM50, as well as candidates of unknown components of this complex. TMT-based quantitative phosphoproteomics revealed many PINK1-regulated phosphorylation sites, such as an autophosphorylation site of PINK1, Ser65 of ubiquitin, and Ser111 of Rab GTPases. Interestingly, several proteins showed both interaction with PINK1 and phosphorylation in a PINK1-dependent manner. These interaction and phosphorylation events were validated by Western blotting and in vitro kinase assays.

Conclusions
These results suggest that PINK1 interacts with and phosphorylates novel proteins, which may play uncharacterized roles in PINK1 signaling.

Keywords
PINK1, Parkinson's disease, IP-MS, TMT, phosphoproteome

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COMBINATORIAL MASS SPECTROMETRY (MS) AND ARTIFICIAL INTELLIGENCE (AI) SUBCLASSIFICATION OF DIFFUSE GLIOMA

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BACKGROUND: Diffuse gliomas are the most common primary brain tumors with variable prognosis. While genomic profiling efforts have identified modest genomic predictors of glioma patient survivals in ~8% of cases, to date large-scale proteomic profiles have not been performed. Similarly, little progress has been made to refine histologic classification and risk stratification of diffuse gliomas and would benefit from artificial intelligence (AI)-based image analysis of glioma biopsies using convolutional neural networks (CNNs). We hypothesize that this combinatorial analysis will improve understanding and prognosis of diffuse gliomas.
METHODS: We utilize MS and CNNs to establish (1) protein and (2) morphometry-based ("phenotypic") predictive diffuse glioma clinical subgroups. Towards AIM1, we apply a developed pipeline utilizing Q Exactive high resolution label-free quantifcation (LFQ) MS to characterize proteomic signatures in a cohort of diverse clinically well-annotated brain tumor specimens (n=50). Towards AIM2, we utilize a CNN-based image analysis for automated brain tumor diagnosis. We are, thus, in a position to leverage histologic analyitical outcomes with glioma proteomic profiles.
RESULTS: Our LFQ MS analytical method is well validated with ~2,500 protein quantifications per tumour sample identifying distinct proteomic-based glioma subtypes (ie. oligodendroglioma, astrocytoma and glioblastoma) based on ~250 changes in protein abundance ($p<0.05$). GOterm based pathway analysis demonstrates that glioma-associated molecualr pathways are perturbed in correct tumour types, providing validation that MS-based proteomic measurements are identifying unbiased proteomic signatures of glioma subtypes. Similarly, training of our CNN using a dataset of 1x106 images of diffuse gliomas result in successful tumour inclusion identification from surrounding non-pathological tissue in novel cases enabling classification of specific glioma subtypes.
CONCLUSIONS: Our combinatorial approach identifies molecular- and image-based glioma subtypes and, thus, has the potential to provide precise and cost-effective clinical prognosis with faster turn-around times than classical neuropathology workflows.

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Proteomics of differentiated cultures of human bronchial epithelial cells exposed to spores of Aspergillus fumigatus

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Background
Aspergillus fumigatus (A. fumigatus) is a ubiquitous filamentous fungus that disseminates in the air through the release of spores. Upon inhalation and binding/internalization by airway epithelial cells, spores may cause an infection in immunocompromised individuals. Our aim was to identify key human host proteins and biological pathways to provide insights into the molecular mechanisms of this dual-organism interaction.

Methods
Differentiated air-liquid interface (ALI) cultures of primary human bronchial epithelial cells (HBECs) were incubated with and without A. fumigatus spores for 6 hours (n=3/group). Proteins were extracted and analyzed by LC-MS/MS using stable-isotope labeling for quantification. MaxQuant software and LIMMA (R package) were used to identify differentially abundant proteins. Pathway enrichment analysis was conducted using DAVID bioinformatic resources.

Results
Of the 2,875 proteins identified, 2,366 were reliably quantified. Of these 2,366 proteins, 222 host proteins were differentially abundant in response to spore exposure (119 up-regulated and 103 down-regulated) (p value <0.05). Negative regulation of transcription, endoplasmic reticulum (ER), unfolded protein binding and chaperone terms were enriched in the 119 up-regulated proteins. Gene ontology terms such as rRNA processing, translation initiation and SRP-dependent co-translational protein targeting to membrane were enriched in the 103 down-regulated proteins.

Conclusion
Shotgun quantitative proteomics identified candidate proteins and pathways to be validated in functional assays as potential mediators of A. fumigatus pathogenesis.

Keywords
Human bronchial epithelium; Aspergillus fumigatus; fungal spores; shotgun proteomics; infection.

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Deregulation of Immunological Pathway in Chronic Schizophrenia Analysed by Quantitative Proteomics

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Background: Schizophrenia is a well characterised mental illness, affecting approximately 1% of the global population. Currently, there is a lack of understanding of immunological pathogenesis in schizophrenia, which is proposed to play a key role in the disease. We hypothesised that proteome profiling of peripheral blood mononuclear cells (PBMC) from chronic schizophrenia cases will be directive in understanding the molecular pathways.

Methods: Blood samples were obtained from 3 chronic schizophrenia patients (on antipsychotic medication) and 3 healthy controls after written informed consent. PBMCs were extracted using histopaque method. Proteins were extracted by sonication in SDS, peptides were prepared using trypsin digestion. The enzymatic digestion was confirmed using SDS PAGE analysis. The peptides were labelled using TMT 6-plex labelling method and labelling was assessed using LC-MS run. LC-MS/MS was done using nanospray liquid chromatography coupled tandem mass spectrometry orbitrap Fusion Tribrid platform. The experiment was run as technical replicates. The MS/MS results were searched against Refseq database (MASCOT and SEQUEST) using Proteome Discoverer V 2.1.

Results: A total of 3450 proteins were reliably identified with ~72% agreement between two replicates. From a combined analysis 154 proteins were found to be down regulated and 136 were upregulated. Pathway analysis showed that T cell activation markers such as LAT, NFATC3, Cofilin 2 etc were significantly down regulated while B cell activation related proteins such as GPCR83, CD79B were upregulated. In addition, the study found significant deregulation of GSK/AKT, apoptosis and energy metabolic pathways.

Conclusions: The study suggests that immunological pathways are possibly an important contributor to inflammation. This continues to be a significant factor in chronic schizophrenia patients despite treatment with antipsychotics. This may have importance in the etiopathogenesis, chronicity and significant treatment implications. This may also explain treatment failure rate reported in the literature.

Keywords: Chronic Schizophrenia, GSK-AKT pathway, Clinical Proteomics.

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Impact Evaluation of Traditional Chinese medicine on Rat liver microsome P450s with Mass Spectrometry

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Background: Most drugs were metabolized through CYP450s and have regulatory roles in CYP450s expression. This constitutes the basis for CYP450s based drug-drug interactions. Traditional Chinese medicine (TCM) is often used in combination; the interaction between TCM is complex, but it lacks systematic in-depth study. Methods: Here, we successfully demonstrated an application of mass spectrometry with Parallelled reaction monitoring (PRM) to quantify eleven liver enzyme CYP450s from the rat models which fed with TCM such as salvia miltiorrhiza, Radix paeoniae alba, monkshood, Shenfu and Panax ginseng. Results: With QconCAT heavy peptides as internal standard, we aimed to assess the effect on CYP450s that caused by the five herbs. The inaccuracy values (RE) and imprecision values (RSD) of QconCAT–PRM were 3.85% and 4%, respectively. The eleven quanified CYP450 isoforms were CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2C6, CYP2C11, CYP2D1, CYP3A1, CYP3A2, CYP17A1 and CYP2E1, with their abundance were 15.12, 14.43, 5.63, 2.37, 6.05, 18.27, 16.24, 4.78, 48.2, 69.32, 66.66 fmol/μg protein in normal rat liver microsome. Salvia miltiorrhiza had inhibitory effect on CYP1A1, CYP2B2 and induced effect on CYP1A2, CYP2B1. Panax ginseng inhibited the expression of the CYP3A2, CYP2C11 and CYP17A1. Monkshood inhibited the expression of CYP1A1, CYP2C6, CYP2C11, CYP3A2 and induced the expression of CYP1A2 and CYP2D1. The expression of CYP2C6, CYP2C11, CYP3A2, CYP17A1 were inhibited by itum carmichaeli, CYP1A1 and CYP2D1 otherwise. And Shenfu had inhibitory effect on the expression of CYP1A1, CYP2C6, CYP2C11, CYP2E1, CYP3A2, and induced effect on the expression of CYP1A2 and CYP3A1. Conclusions: Our research provides the method support and the theory basis for drug compatibility in practice and avoids adverse drug reactions.
Keywords: CYP450s ; Parallelled reaction monitoring (PRM); Traditional Chinese medicine (TCM)

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An Automated and Integrated SWATH-MS informatics pipeline for clinical proteomics

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Background
SWATH-MS is a high throughput DIA LC/MS technique to rapidly and reproducibly identify and quantify proteins across multiple proteolysed biological samples. Despite early successes using this technique, the scale of SWATH-MS data and the nascent state of computational tools for interrogation of SWATH-MS data have provided new challenges, with multiple steps of per-sample analysis required, including translation from vendor formats, protein identification and quantitation, and confidence scoring. This multi-step pipeline requires detailed knowledge of the all steps of the analysis performed, with small studies potentially taking many weeks of human and computational time despite the use of powerful workstations, and sometimes resulting in repeated experiments yielding different results.

Methods
A system was developed using Apache Taverna, a scientific workflow engine, and a high-memory HPC at the University of Manchester to perform OpenSWATH-based analysis, performant in a clinical environment. A web-based graphical user interface allows the submission of SWATH-MS data with minimal knowledge of the experimental protocol, to follow a user-defined workflow, and producing a provenance file describing the steps performed and the variables used.

Results
A 20-fold increase in throughput was demonstrated, with 100 SWATH samples analysed from a single study in a single 24 hour period. Results were highly reproducible, and provenance output stored all variables used. The various software packages and example workflows to perform SWATH analyses are made available as a runnable virtual appliance for integration with other institutions' SGE clusters.

Conclusion
The automated, integrated pipeline supports clinical applications of SWATH-MS in a high-throughput setting. It provides a permanent record of experimental provenance, and supports rationale and repeated interrogation of the same mass spectrometry data with different parameters. We believe the leveraged power of HPC systems and the simplified user interfaces could significantly assist researchers using SWATH-MS to define clinical proteomic biomarkers.

Keywords: SWATH-MS, Taverna, Automation

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iProX: the integrated Proteome Resources in China

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Sharing of research data in public repository has become an irreversible trend in academic. While with the accumulation of massive data, the requirements of network bandwidth and storage grow rapidly. The ProteomeXchange consortium implement a mode of centralized meta-data and distributed raw-data management, which promotes the data sharing effectively. To support the Chinese Human Proteome Project and facilitate open access of proteome data, the integrated proteome submission system (iProX, www.iprox.org) was built, as a public platform for collecting and sharing standardized meta-data and raw-data obtained from proteomics experiments.

iProX repository employs a web-based process of proteome data submission. Also, iProX makes extensive use of controlled vocabularies and ontologies in the PRIDE OLS system to annotate the entries of proteomics datasets. iProX is fully compatible with the data-sharing policies and rules proposed by ProteomeXchange. Registered users can submit their proteomics datasets to iProX in public or private way. All released datasets are freely accessible to public (including anonymous users) while the private datasets can only be accessed by authorized users. By the end of July 2017, 285 datasets have been submitted to iProX, accumulating a total of 40 TB data.

Keywords

iProX / data sharing / data submission / proteomics

Reference

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The iMOP initiative and its roles in a Biology and Disease driven Human Proteome Project

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The Human Proteome is nearly fully mapped and will provide a knowledge base to accelerate our understanding of how proteins and protein networks can affect human health and disease. However, providing solutions to human health challenges will likely fail if insights are exclusively based on studies of human samples and human proteomes. In recent years, it has become evident that human health depends on an integrated understanding of the many species that make human life possible. These include the commensal microorganisms which are essential to human life, pathogens and food species as well as the classic model organisms that enable studies of biological mechanisms. The Human Proteome Organization (HUPO) initiative on multi-organism proteomes (iMOP) works to support proteome research undertaken on non-human species which remain widely under-studied compared to the progress in human proteome research. This perspective argues the need for further research on multiple species that impact human life. We also present an update on recent progress in model organisms, microbiota, and food species, address the emerging problem of antibiotics resistance, and outline how iMOP activities could lead to a more inclusive approach for the human proteome project (HPP) to better support proteome research aimed at improving human health and furthering knowledge on human biology.

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Efficient micro-scale basic reverse phase peptide fractionation for global and targeted proteomics

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Analysis of small biological samples would benefit from an efficient micro-scale fractionation strategy that minimizes sample handling, transfer steps and accompanying losses. Here we describe a micro-scale basic reverse phase liquid chromatographic (bRPLC) fractionation method that offers high reproducibility and efficiency for peptide mixtures from small (5-20 µg) samples. We applied our platform to detect differentially expressed proteins from lung tumor cell lines that are sensitive (11-18) and resistant (11-18R) to the tyrosine kinase inhibitor erlotinib. Label-free analyses of 5-20 µg samples yielded identifications of approximately 3,200 to 4,000 proteins with coefficients of variation of 1.9-8.9% in replicate analyses. iTRAQ analyses produced similar protein inventories. Label free and iTRAQ analyses displayed high concordance in identifications of proteins differentially expressed in 11-18 and 11-18R cells. Micro-bRPLC fractionation of cell proteomes increased sensitivity by an average of 4.5-fold in targeted quantitation using parallel reaction monitoring for 3 representative receptor tyrosine kinases (EGFR, PGFRA, and BMX), which are present at low abundance in 11-18 and 11-18R cells. These data illustrate the broad utility of micro-bRPLC fractionation for global and targeted proteomic analyses.

Kyewords: Micro-scale fractionation, Proteomics

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Pervasive co-expression of spatially proximal genes is buffered at the protein level

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Genes are not randomly distributed in the genome. In humans, 10% of protein-coding genes are transcribed from bidirectional promoters and many more are organised in larger clusters. Intriguingly, neighbouring genes are frequently coexpressed but rarely functionally related. Here we show that coexpression of bidirectional gene pairs, and closeby genes in general, is buffered at the protein level (Kustatscher et al, accepted July 2017, Mol. Syst. Biol.). Taking into account the 3D architecture of the genome, we find that co-regulation of spatially close, functionally unrelated genes is pervasive at the transcriptome level, but does not extend to the proteome. We present evidence that non-functional mRNA coexpression in human cells arises from stochastic chromatin fluctuations and direct regulatory interference between spatially close genes. Protein-level buffering likely reflects a lack of coordination of post-transcriptional regulation of functionally unrelated genes. Grouping human genes together along the genome sequence, or through long-range chromosome folding, is associated with reduced expression noise. We conclude that non-functional mRNA coexpression is far more common than previously thought. Generally, this does not hamper cell fitness as post-transcriptional regulatory mechanisms enforce functional coexpression while dampening non-functional coexpression. Our observations suggest that evolution of human genome organisation is driven by noise reduction, which is an hypothesis initially made in yeast. The large presence of non-functional co-expression of genes at the transcript but not protein level has implications for the fields of transcriptomics and proteomics when screening for functional links between genes.

Keywords: Genome organisation / Gene expression noise / Regulatory interference / Proteomics / Transcriptomics

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Proteomic investigation supporting behavioural characterization of recovery upon DHA treatment of Spinal Cord Injury (SCI)

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Background: Traumatic spinal cord injury (SCI) is one of the most difficult and urgent health challenges in neurodegenerative medicine. Animal models of SCI try to mimic clinical situations however translation of experimental findings to the human condition is far from being effective.
Proteomic can be a substantial support for researchers in the field to validate the biochemistry of behavioral observation and to charachterize potential prognostic biomarkers.
In this work we performed a shotgun proteomic study on a mouse model of SCI upon post trauma treatment with Docosahexaenoic acid (DHA) to charachterize the model and the putative translational implications for a novel therapeutic approach to functional recovery.

Methods: SCI was performed in female CD1 mice. DHA or saline, were intraperitoneally administered within 1hr post trauma and once /day for the next 5 days.
Functional recovery was evaluate by tail flick and Basso Mouse Scale score.
A label-free shotgun proteomic analysis was then pursued to analyse proteome modulation upon treatment with DHA in the injured spinal cord 7 days post trauma. To characterize structural and biochemical changes immunohistochemistry and antibody array assay were performed 7 and 30 days after trauma.

Results: Proteomic results in SCI animals and their bioiformatic analysis unravel a modulation of proteins involved in metabolic and inflammatory pathway, in agreement with IHC and immunoenzymatic experiments.
Moreover these data support the hypothesis that DHA treatment can sustain the regenerative process of the injured tissue.

Conclusions In this study we supported with a proteomic analysis the investigation of reliable and sensitive mouse model of SCI, and the effect of DHA subministration on functional recovery. Proteomics, in agreement with behavioural, histochemical and immunioenzymatic observations, show that DHA is able to restore complete motor function as well as nociceptive sensitivity in paraplegic mice

Keywords: proteomic, SCI, regeneration, paralysis, behavior

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Proteomic analysis of single cell clusters using laser capture microdissection

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Background
An inherent problem with the analysis of 'bulk' tissue by traditional proteomics methods is that the cellular heterogeneity of a tissue is often unaddressed. This heterogeneity results in averaging of the signals generated by the constituent cells and the data are dominated by those cells that are the most abundant. Isolating cells of a single type by laser capture microdissection alleviates the signal averaging caused by measuring a mixed population of cell types. Here we developed a simple, quantitative sample preparation method for sub-1000 cells isolated by laser capture microdissection.
Methods
We used laser capture microdissection to isolate varying numbers of Purkinje cells from histological tissue slices of cerebellum obtained from post-mortem human donors into an adhesive coated microcentrifuge tube lid. Captured proteins were proteolytically digested with trypsin and the resulting peptides were analysed by LC-MS/MS on an Orbitrap Fusion Lumos. Data files were processed and analysed with MaxQuant and Perseus.
Results
We were able to reproducibly detect over 2000 proteins from only 200 Purkinje cells isolated from histological tissue slices. The overlap in protein identifications was 80 % between two replicates from different tissue slices within the same donor. The proteomes generated were deep enough to detect several proteins involved in Purkinje cell development and function along with almost 600 proteins involved in general neurological cell processes. The method also shows good quantitative reproducibility between replicates (Pearson Correlation Coefficient = 0.90).
Conclusions
The coupling of laser capture microdissection and proteomics is a powerful technique capable of generating reproducible, quantitative proteomes of sufficient depth from a single type of cell isolated from a mixed population, which has the potential to yield unbiased information about mechanisms of cell fate specification and degeneration in the human brain.
Keywords
Laser capture microdissection; ultrasensitive proteomics; quantitative proteomics; neuropathology; single-cell isolation

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Proteomic analysis of plasma-derived extracellular vesicles in breast cancer PDX models

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Background
Extracellular vesicles (EVs) are released by cancer cells into their environment and are implicated in cancer progression and metastasis. There has been a growing interest to search for disease-relevant biomarkers carried by EVs. However, isolating tumour-specific EVs from plasma remains technically challenging.

Methods
In order to detect EV proteins of tumour origin in vivo, we made use of patient-derived xenografts (PDXs) of human breast cancer that allowed us to discriminate species-specific proteins. We purified EVs from PDX mouse plasma by size-exclusion chromatography. For validation, we mixed wild-type mouse plasma with a gradient series of SILAC-labelled EVs derived from MCF-7 breast cancer cell line. Samples were measured by high-resolution mass spectrometry.

Results
We detected 759 to 2399 proteins per PDX EV sample from ~100 µl of plasma. Of these proteins, only a small proportion were identified with at least one human sequence-specific peptide. However, we observed an increase in signal intensity of human-mappable peptides compared to negative controls. This suggests that the proportion of tumour-specific EV proteins is higher than reflected by the number of human-specific peptides.

Conclusions
In conclusion, tumour-derived EVs could be isolated from the plasma of breast cancer PDX models. We are currently working on a statistical model to infer identification of tumour EV proteins based on the mappability and signal intensity of detected peptides.

Keywords
breast cancer; extracellular vesicle; PDX; plasma

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Combined Top-down and Bottom-up Proteomics using
Capillary Electrophoresis–Mass Spectrometry

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Background:
Bottom-up proteomics has been widely used for resolving the sequence of a single protein or components of protein complexes. Bottom-up approaches for single protein characterization enable the localization of PTMs via digested peptide; however, the stoichiometry of PTMs are not able to be resolved easily. Top-down approaches have proved to be a better solution for understanding combinatorial PTM profiles. A combined top-down and bottom-up proteomics workflow is a promising solution for comprehensive protein characterization. Current analytical strategies mainly rely on LC-MS. However, protein separation used for top-down and peptide separation used for bottom-up require different LC platforms; therefore, the combined workflow using LC-MS is usually time consuming.

Methods:
In this study, we introduce a new platform of CE-MS for proteomics research. On this platform, intact proteins and peptides can be monitored on the same microfluidic CE chip, and top-down and bottom-up analysis can be combined on one CE-MS platform. The whole workflow only takes 18 min including 3 min for intact analysis, 3 min for top-down analysis and 8 min for bottom-up analysis.

Results:
Two model proteins have been investigated on this platform. For cytochrome c, 56% residue cleavage from top-down and 97% sequence coverage from bottom-up was observed with high-energy collisional dissociation (HCD). In the case of carbonic anhydrase, 19% residue coverage and 97% sequence coverage was detected. Oxidation and deamidation are able to be monitored. It demonstrated the capability for comprehensive top-down and bottom-up analysis on this CE-MS platform within 18 min.

Conclusions:
To demonstrate the utility of this combined platform, we utilized recombinant KRAS and PD-L1, expressed in HEK293 cells to investigate disease relevant PTMs. The localization and stoichiometry of multiple PTMs are expected to be acquired on this CE-MS platform with combined workflow in a timely manner.

Keywords:
Top-down, Bottom-up, Capillary Electrophoresis-Mass Spectrometry

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Systematic analysis of drug-protein targets in native biological systems

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Protein binding to drug molecules is a powerful mechanism to modulate protein function with relevant consequences on the physiology of the cell and it is commonly used for the discovery and optimization of new therapeutics. Protein target-based screens are generally performed on purified protein samples in vitro and do not take into account the contributions of cellular compartmentalization, protein-protein interactions and post-translational modifications, all factors that can affect the activity of drug compounds in cellular environments. Thus, the discovery of ligand protein ligand-interactions in an unbiased and systematic way is extremely challenging.

In order to address this open question, we are applying a novel chemical proteomics approach based on limited proteolysis (LiP) and latest generation mass spectrometry of complex samples, which has been recently presented by our group (Feng et. al, 2014). The LiP strategy relies on proteases with broad specificity applied under controlled conditions to a proteome extract, such that the structural features of the protein substrates determine the proteolytic sites. We search for differential proteolytic digestion profiles to infer ligand-induced conformational changes and identify unknown drug-protein targets and their binding sites.

As a proof of principle, microbial proteomes are analyzed in the presence and absence of drug small-molecules to find proteins that change proteolytic patterns upon administration of ligand compounds. The results recapitulate a number of known drug protein interactions and lead to the identification of novel protein-drug interactions. This work holds promise for the unbiased analysis of unknown molecular targets of drugs for many proteins at the same time directly in their native cellular environment.

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Evaluation of dimethylated arginine containing peptides by LC-MS/MS with Electron Transfer Dissociation

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Background

Protein arginine methyltransferases (PRMTs) are essential enzymes for cell viability, and elevated PRMT levels play a role in cancer cell growth and metastasis. PRMTs catalyze the post-translational modification (PTM) of protein arginine residues with monomethylation and asymmetric or symmetric dimethylation. These isobaric species are difficult to differentiate by mass spectrometry, with most methods relying blindly on the specificity of antibodies. By utilizing Electron Transfer Dissociation (ETD), commercially available antibodies can be evaluated for specificity, and enhanced accuracy of identification of protein methylarginines can be obtained.

Methods

Lung cancer cell lines are treated with PRMT inhibitors, digested with trypsin, and enriched for symmetric or asymmetric dimethyl arginine containing peptides by immunoprecipitation with antibodies from Cell Signaling Technologies. The enriched peptide elutions are evaluated using HPLC-MS/MS on a Thermo Orbitrap Fusion™ Tribrid™ instrument. A combination of database searching with Byonic™, label-free relative quantitation with Proteome Discoverer 2.2™, and manual evaluation of MS/MS spectra to confirm the specificity of the antibodies will be used to validate the results.

Results

Methylated arginine residues inhibit tryptic cleavage, yielding longer and highly charged peptides, optimal targets for ETD. Peptides detected with the method range in charge states from +3 to +10, indicating multiple dimethylated arginines on a given peptide. ETD yielded side chain losses that were characteristic of the symmetry of the modifications, along with site localizing fragments. In comparison, collisionally activated and/or higher energy collisionally dissociated spectra of these highly charged species are difficult to interpret or are not even assigned by the search algorithm. Two RNA binding proteins, with multiple dimethylated arginine residues, will be used to demonstrate the methodology.

Conclusions

Electron Transfer Dissociation is the optimal fragmentation technique for highly modified and charged peptides, capable of adding an enhanced level of accuracy to methylated peptide identification.

Keywords

Methylation, Arginine, Electron Transfer Dissociation

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Myristoylation of human FMN2 protein causes individual localization pattern and function

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Background
Formin-2 (FMN2) is a novel member of tumour suppressor protein, which is up-regulated by several stress conditions, including oncogene activations, DNA damage, and hypoxia shock, and up-regulates cycline dependent kinase inhibitor 1A (CDKN1A, p21) at the protein level by prevention of proteasome degradation pathway (Yamada et al., 2013). Lipid modifications of proteins are broad in nature and play a significant role in several biological processes. We discovered that human FMN2 has 2 isoforms, and one of the N-terminal region has a lipid modification, known as myristoylation.

Method
We established inducible stable cell lines of these isoforms with GFP-tags and they showed different localization patterns in the U2OS cells. The Long form of FMN2 protein (FMN2-L) localized mainly in the cytoplasm and partially in nuclear, and the FMN2 isoform with myristoylation (FMN2-M) localized in the plasma membrane of the cells. We demonstrated SILAC-IP experiments using those inducible cell lines by in gel digestions, and mass spectrometry analysis was performed using the Thermo Scientific Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. The RAW data produced by the mass spectrometer were analysed using the quantitative proteomics software MaxQuant. Data analysis was performed on Gene Ontology enRIchment anaLysis and visuaLizAtion tool (GORILLA, http://cbl-gorilla.cs.technion.ac.il/).

Result
We discovered that the FMN2-M is binding to several membrane and signal transduction proteins more than the FMN2-L in the potential binding partners detected by mass spectrometry analysis. With multiple possibilities, we focused on the epidermal growth factor receptor (EGFR) and downstream signal transduction proteins, and found the over expression of FMN2-M inhibits phosphorylation and/or expression of extracellular-signal-regulated kinase (ERK) protein.

Keyword
FMN2, cancer, myristoylation, Lipid modification, EGFR, ERK

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Quantitative profiling of post-translational modifications by labeling-based mass spectrometry approaches

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Background:
Protein post-translational modifications (PTMs) play critical roles in diverse biological processes. LC-MS/MS-based isotope labeling strategies such as SILAC, iTRAQ and TMT has gained popularity to identify and quantify PTMs and their dynamics. The challenge of PTM quantitation by data-dependent acquisition is partially caused by the presence of missing values of low-abundant peptides, which decreases accuracy and sensitivity. To recover quantitation information of low-abundant peptides, a new algorithm was developed for PTM analysis. Initial test showed increased sensitivity and accuracy for PTM profiling across complex biological samples.

Methods:
First, peptide features were extracted from MS1 data. MS2 data were searched against databases for peptide (unmodified and modified) identification. Alignment of multiple LC-MS runs was used to increase sensitivity and accuracy. Missing data were replaced by paired feature intensities by matching within tight retention time, isotopic envelops and mass windows to enable quantification without identification.
Second, for each modification site identified, peptides that contain the PTM site are categorized into either unmodified or modified group based on whether the specific site is confidently modified.
Third, unmodified and modified peptide intensities in different samples/labeling channels are calculated based on: (1) for precursor ion labeling (e.g. SILAC): summed peptide feature intensities of unmodified and modified groups for different labeling channels separately; (2) for reporter ion labeling (e.g. TMT and iTRAQ): summed peptide feature area from MS1 XICs of unmodified and modified groups that are distributed to different samples/labeling channels respectively based on ratios of their reporter ion intensities. Furthermore, PTM quantification results can be normalized by protein ratio estimations.

Results:
The algorithm was implemented in PEAKS Studio software and tested. The phospho-tyrosine quantification results from a SILAC phosphoproteome study (PMID: 28111465) showed high inter-replicate reproducibility.

Conclusions:
The new algorithm provides PTM quantification with high sensitivity and accuracy.

Keywords: PTM, SILAC, quantification, phosphorylation

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Abnormal tear proteomic profile in patients with traumatic vegetative state

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Background: Vegetative state is a complex condition with unclear mechanisms and limited diagnostic, prognostic and therapeutic methods. In this study, we aim to explore the tear proteomic profile in patients with traumatic vegetative state (TVS) and identify potential diagnostic markers using tears – a body fluid can be collected non-invasively.
Methods: In discovery phase, tear samples collected from 16 TVS patients and 16 normal subjects were analyzed using iTRAQ quantitative proteomics. Validation was carried out using tears from 50 TVS patients and 50 normal controls (including samples used in discovery phase) using ELISA.
Results: Among 1080 identified tear proteins, 58 and 16 proteins were found to be up- and down-regulated in TVS patients as compared to controls. Bioinformatics analysis revealed that the differentially expressed proteins were mostly involved in wound response and immune response signaling pathways. The validation results showed that a 7 protein-panel has high discrimination ability for TVS (AUC = 0.999). Conclusions: In summary, the altered tear proteomic profile identified from this study provided the bases for potential tear protein markers for diagnosis and prognosis in TVS and may also suggest novel insights into the mechanisms of TVS.
Keywords: Trauma, vegetative state, tears, iTRAQ proteomics,

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Bioorthogonal labeling of human prostate cancer tissue slice cultures for glycoproteomics

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Sialylated glycans are found at elevated levels on many types of cancer and have been implicated in disease progression. However, the specific glycoproteins that contribute to cancer cell surface sialylation are not well characterized, specifically in bona fide human disease tissue. Metabolic and bioorthogonal labeling methods have previously enabled enrichment and identification of sialoglycoproteins from cultured cells and model organisms. Here we report the first application of this glycoproteomics platform to human tissues cultured ex vivo. Both normal and cancerous prostate tissues were sliced and cultured in the presence of the azide-functionalized sialic acid biosynthetic precursor Ac_4ManNAz. The compound was metabolized to the azido sialic acid and incorporated into cell surface and secreted sialoglycoproteins. Chemical biotinylation followed by enrichment and mass spectrometry led to the identification of glycoproteins that were found at elevated levels or uniquely in cancerous prostate tissue. This work therefore extends the use of bioorthogonal labeling strategies to problems of human clinical relevance.

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Mevalonate pathway inhibitors as potential therapeutic agents for Hepatocellular carcinoma: an in vitro study

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Hepatocellular carcinoma (HCC) is fifth most common cancer occurring globally with 560,000 new cases each year. Incidence of HCC in Pakistan is approximately 10.5%. Due to late diagnosis, mortality rates are very high with survival rate of only 3.5%. In advance stages, palliative therapy is the only option that serves to improve quality of life. Till date, limited options for chemotherapy for HCC are available with modest improvement in survival rates. Current study was designed to investigate apoptotic effect of FDA approved drugs Alendronate (ALN), 5-Azacytidine (5`-AzaC), FTI-277 and their combinations along with changes in gene and protein expression in Huh-7 cell lines. The drugs and their combination were found to induce cytotoxicity in time and dose dependent manner. Gene expression was examined using qPCR for FDPS, FDFT1, RAB11A, RND3, CASP7, LZTS1, DNMT1, Prx2 and GSTP1. For proteins expression studies, combination of two dimensional gel electrophoresis and Mass spectrometric analysis were employed and differentially expressed proteins were identified. We identified twenty proteins from all (single and combination) treatment groups belonging to various biological functions such as signal transduction, metabolism, apoptosis, cell growth and protein folding. Uniquely identified proteins in this study include Protein dicaudal D homolog1 isoform1, Interleukin-7, Ras association domain-containing protein 7, V(D)J recombinant activating protein 2 and Nuclear factor erythroid 2-related factor 2. Our study revealed combination therapy to be more effective with greater efficacy than single dose treatment. Use of proteomics not only offers insight in understanding mode of action of different drugs, it is also likely to be beneficial for the development of novel therapeutic combination for end stage treatment of HCC.

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Cell surface antigen-antibody microarray as a tool for detection macrophages polarization

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Tumor cell invasion and metastasis are the main reason of increased morbidity and mortality among cancer patients world wide. Recent advances in cancer research have shown that tumor associated macrophages (TAM) play a substantial role in controlling and accelerating tumor cell metastasis. Macrophages generally attain an antimicrobial and anti-tumor activity within tissues. However, during the process of tumorigenesis many of the soluble factors released within the tumor micrenvironement shift the innate protective function of macrophages in a process known as macrophage polarization. Macrophages can polarize into either M1 macrophages, which are immuno-supportive, or M2 macrophages, which are immuno-suppressive/cancer promoting cells. The differentiation between M1 and M2 macrophage phenotypes has always been hampered by the lack of a sensitive and specific cell surface antigen that can help characterization of the particular cell type. In this report, we an antibody microarray of over 300 antibodies targeting cell surface antigens was used to characterize specific markers for M1 and M2 macrophages. The identified markers were further elucidated by FACS analysis and immunofluorescence. The study provides a new set of cell surface antigens that can help detection the specific pattern of macrophage polarization.

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Combined targeted and shotgun proteomics analysis in saliva for verification of markers of aggressive periodontitis

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Background:
Aggressive periodontitis affects severely the tooth supporting (periodontal) tissues, which is characterized by a rapid progression and early life manifestation. There are still huge challenges in performing early diagnosis of the disease, particularly with the lack of appropriate molecular diagnostic methods. Saliva is a non-invasive reservoir of clinically relevant biomarkers with the potential to diagnose early and monitor the state of ongoing disease. Despite several protein markers being enlisted over the years, none of them has been incorporated into daily dental practice as a clinical assay. The main reason is lack of validation and verification steps in larger cohorts due to unavailability of validated antibody-based assays for multiplexing purposes.

Methods:
We have followed two main mass-spectrometry techniques to identify biomarkers of aggressive periodontitis in human saliva. Firstly, the shotgun proteomics study with case-control design (n=67) conducted to dissect comparative saliva proteome along the different disease stages from health, gingivitis, aggressive to chronic periodontitis. Secondly, we have conducted similar case-control study with an independent large series of patient samples (n=80) to verify sixty-four candidate markers by selected-reaction monitoring (SRM)-based targeted proteomics approach.

Results:
Using shotgun quantitative proteomics, we detected hundred proteins displaying significant different abundance changes between the aggressive periodontitis and the healthy group. These proteins showed significant enrichment of multiple cellular and molecular processes such as cytoskeleton rearrangement and immune response. Further targeted proteomics experiments performed on independent cohort of saliva samples confirmed that twelve proteins were able to discriminate between the aggressive periodontitis and the healthy individuals with maximum area under the receiver operating curve greater than 0.82.

Conclusions
The discovered proteins will expand the pool of biomarker candidates that can be used to develop a clinical assay for the early detection of aggressive periodontitis.

Keywords:
Saliva, targeted proteomics, shotgun proteomics, biomarker verification

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Comprehensive analysis of human protein N-termini enables assessment of various protein forms

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Background;
Various forms of protein (proteoforms) are generated by genetic variations, alternative splicing, alternative translation initiation, co- or post-translational modification and proteolysis. Different proteoforms are in part discovered by characterizing their N-terminal sequences.

Methods;
We introduce an N-terminal-peptide-enrichment method, Nrich. Filter-aided negative selection formed the basis for the use of two N-blocking reagents and two endoproteases in this method. Proteins were labeled with an N-blocking reagent, digested with an endoprotease, and peptides with newly generated free N-termini were removed using NHS-bead to leave N-terminome.

Results;
We identified 6,525 acetylated (or partially acetylated) and 6,570 free protein N-termini arising from 5,727 proteins in HEK293T human cells. The protein N-termini included translation initiation sites annotated in the UniProtKB database, putative alternative translational initiation sites, and N-terminal sites exposed after signal/transit/pro-peptide removal or unknown processing, revealing various proteoforms in cells. In addition, 46 novel protein N-termini were identified in 5' untranslated region (UTR) sequence with pseudo start codons.

Conclusions;
Our data showing the observation of N-terminal sequences of mature proteins constitutes a useful resource that may provide information for a better understanding of various proteoforms in cells.

Keywords;
N-terminome, negative selection, N-acetylation, UTR

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Phosphoproteomic analysis of murine synaptosome in sleep deprivation

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Background
Sleep has been ascribed a critical role in cognitive functioning, with implication in synaptic homeostasis and long-term memory. Acute and chronic stress and total sleep deprivation (SD) impairs memory consolidation, attention, working memory and perception. Despite the wide scientific interest on the effects of sleep on the synapse, there is a lack of systematic investigation of sleep-related changes in the synaptic proteome and phosphoproteome.
Methods
We isolated parietal cortical synaptosomes of rats after 8 h of total SD by gentle handling and 16 h after the end of deprivation to investigate the short- and long-term effects of SD. Samples were digested and phosphopeptides were enriched with TiO2.
Results
Along with protein abundance alterations, data of phosphorylation changes in synaptosomes is now available using 6 animals/group and all sample preparation done in replicates. Although most protein's abundances don't change significantly in SD and recovery sleep, we identified several hundred phosphorylation events that vary between groups. In total 1895 phosphorylation sites were identified.
Conclusions
The data was first analyzed by GO enrichment analysis and pathway analysis. Several altered proteins were found to be involved in synaptic strength regulation, neurotransmitter vesicle trafficking and energy metabolism. The identified protein signaling pathways show the sleep-related synaptogenesis and molecular maintenance. We show that in sleep deprivation, phosphorylation based signalling activates the thyroid hormone synthesis and insuline secretion as a whole body response to sleep deprivation and stress.

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Proteome analysis of exhaled breath condensate before and after space flight

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Background

As a part of development of medicine for extreme situations, we determined, for the first time, the protein composition of the exhaled breath condensate (EBC) of cosmonauts before and after long-lasting orbital flights on the International Space Station (ISS).

The purpose of the experiment "Protocon" was the study of the EBC proteome and metabolome changes for Russian cosmonauts, who are members of the main crew of International Space Station (ISS). We started working on this topic in April 2013, with expedition #35 to the ISS. At the moment we have 13 complete sets of samples (before/after flight to ISS).

Methods

The analysis was performed by electrospray ionization ion cyclotron resonance mass spectrometry. The pattern of variation of the protein composition of the exhaled air during preflight preparation, at the time of landing, and seven days after landing was elucidated.

Results

Forty-four proteins that appeared in the exhaled breath condensate of the cosmonauts after a long orbital flight and landing and were absent before the flight were detected. These proteins included the immune response proteins, indicating cell and blood vessel damage. Several protein markers of early lung cancer (POTEE, Cep290, and TBC1D1) were found in several EBC samples of crew members post flight, while these markers were absent from the preflight samples and control samples collected from the crew members half year after landing.

Conclusions

A dynamic observation of changes in EBC composition revealed a dramatic increase in the amount of exhaled proteins at landing. The appearance and subsequent elimination of the cancer markers may be indicative of both potential risk for cancer due to spaceflight factors and overloading (stress and radiation) and the human body potential to restore the normal functions and to prevent cell malignant transformation.

Keywords

Spaceflight; proteomics; extreme conditions; human proteome; exhaled breath condensate

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Bridging the 'translation gap': A prospective study to validate biomarker panels predictive of prostate cancer

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Background

There is a clear unmet need for biomarkers to assist the diagnosis and management of prostate cancer. Omics technologies measure large numbers of analytes. Machine learning on such data in small cohorts creates a significant risk of "overlearning", resulting in promising discoveries that fail to validate or are badly calibrated.

Methods

Our discovery systems were extended via SOPs and statistical process control to provide high quality biomarker measures, which allowed us to validate the marker panels without the need to translate into a targeted assay, thus bridging the 'translation gap'.

A prospective clinical trial, ISRCTN52361806, was initiated to validate the panels. The primary end-point is demonstrating a significant improvement in predicting the outcome of first prostate biopsy over PSA.

A previous clinical discovery study was used to derive the test analytes and to calculate the target population cohort size.

Biosignature panels can suffer from calibration issues when moving from a discovery cohort to a targeted clinical population. This validation study was split into two parts each of sufficient size to deliver the primary endpoint at the desired power and significance thresholds.

Part 1 data can then be used to explore calibration between the discovery and validation cohorts, define a recalibrated version of the original signature and define additional novel protein signatures, all for blind testing on samples from part 2.

Results

In Part 1 blind testing, the protein biosignature demonstrated a significant improvement in predicting prostate biopsy outcome over current practice. Implementation in the clinic could reduce unnecessary prostate biopsies by up to a fifth.

Conclusions

Hypothesis free discovery of marker panels can move on to successful blind clinical validation under tightly controlled conditions -- bridging the 'translation gap'.

Keywords:
Prostate, Biomarkers, Validation

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Accurate prediction of retention time shift for isotopic-labeling based protein quantification

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Background

Despite the advantages such as accurate quantity ratios and reduced technical bias over other approaches, isotopic-labeling based protein quantification approaches (e.g., SILAC) have insufficient multiplexities (≤3-plex) for most biological applications. The use of multiple deuteriums is one possible option to increase multiplexity but is limited due to the retention time (RT) shift between deuterated peptide ion and its counterpart.

Methods

We developed a machine-learning based RT shift prediction method. For training, we prepared HeLa lysate samples labeled by in-house-designed reductive di-ethylation 6-plex (DE6-plex) labeling with the input ratio 1:1:1:1:1:1. The labeled peptides contain up to dozens of deuteriums. The samples were analyzed using Q-Exactive, then only high quality XICs were selected with annotated peak RTs in automated fashion. The relevant features for prediction, such as the number of deuteriums in ion and relative retention time of ion, were selected empirically. The nu-SVR algorithm was used for predictiction.

Results

For benchmark, we used 10-fold cross validation to measure the pearson correlation coefficient (PCC) and the root mean square error (RMSE) between predicted and measured RT shifts. PCC was over 0.8 and RMSE was only 1/10 of the average XIC span. We also observed that individual feature is quite weakly correlated to the RT shift; the strongest feature had PCC of only 0.47. This shows that our method achieves such accurate prediction by combining weak features. Lastly, we adopted our prediction method for the quantification of DE6-plex labeled sample. The quantification variance and bias were significantly less than without our method.

Conclusions

We introduced a machine-learning based method enabling accurate prediction of the RT shift due to deuteriums. We anticipate that our method could facilitate isotopic-labeling based protein quantification at higher multiplexity. Our method is adopted for the tool EPIQ (Epic Protein Integrative Quantification).

Keywords:deuterium effect;retention time shift;isotopic-labeling;protein quantification

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Direct detection of stop codon read-through events using proteomics in Drosophila melanogaster

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Background

Stop codon read-through (RT) occurs when translation fails to terminate at the normal signal, the ribosome decodes the stop codon with a near-cognate tRNA, and translation continues to a secondary, downstream stop signal. Previous studies using phylogenetics and ribosome profiling have defined datasets of 638 strong RT candidates in the model metazoan, Drosophila melanogaster. Although many of these predicted events would result in functionally relevant protein extensions, few have been directly validated by proteomic means.

Methods

We developed an informatic approach to extend protein sequence databases to include predicted RT candidates, which considers all possible RT events and generates the attendant amino acid insert. After validation of the method with simulated stop codon cases in known MS-detectable peptides, we attempted to detect the 638 predicted D.melanogaster RT candidates, using 36 mass spectrometry datasets covering over 20M spectra from different developmental stages and tissues.

Results

In total, 315 peptides from RT sections of 117 proteins were detected, the largest validated set of RT events in a model system. The majority of RTs were detected in head samples with up to 45 RT proteins observed in a single sample. Although this is a substantial number, MS evidence was observed for fewer than 20% of the predicted RTs.

Conclusions

We present the first direct proteomic evidence validating 117 RT events in Drosophila. Despite the previous predictions of widespread RT, we considered why fewer than 20% of candidates were validated. One possibility is the observed bias towards samples containing brain (e.g. heads), which suggests some tissue specificity. Additionally, many candidate RT peptides may not be routinely detectable, either from poor cleavage context, poor ionisation properties, and/or low abundance of the parent protein or RT isoform. Finally, stop codon readthrough may not be as pervasive as previously predicted.

Keywords: Proteomics, read-through, translation

898

Protein iodination in thyroid function and regulation

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Background
Thyroid is an important endocrine glands in human. Iodide uptake and thyroglobulin iodination are crucial for thyroid hormone synthesis. Thyroid function is regulated by TSH (Thyroid Stimulating Hormone), but is also modulated by the concentration of iodide in blood. Although studied for many years, only indirect and partial surveys of protein iodination were reported. In this work, we showed the identification of iodination sites on thyroglobulin and other proteins using mass spectrometry.
Methods
A shotgun proteomic approach was used to semi quantify the level of thyroglobulin iodination in rats treated with different diets. Peptide identifications were performed using liquid chromatography coupled with Orbitrap QExactive Plus spectrometer. In addition, we study in a no thyroidal cell line the putative iodination of proteins other than thyroglobulin using different strategies: radioactive labelling, immunoprecipitation and high-resolution mass spectrometry (Orbitrap Fusion Lumos).
Results
We identified around 30 iodinated tyrosine sites on thyroglobulin. Our results indicate that their iodination level responds to changes in iodine diet or to the presence of thyroid inhibitors. The determination of these iodination peptides provides potential biomarkers for the characterisation of thyroid dysfunctions. In addition, we reported for the first time the identification of iodination proteins other than thyroglobulin that could be involved in thyroid regulation by iodide.
Conclusions
Together, these results show the feasibility of the semi-quantification of thyroglobulin iodination and the detection of low-abundant iodination sites. They are of significant interest for the diagnostic of thyroid dysfunction. Furthermore, our work gives new insights into the understanding of thyroid regulation mechanism, which could partially be controlled by iodinated proteins.
This work was supported by the 'Commissariat General a l'Investissement' ('RSNR' program) and the 'Agence Nationale de la Recherche' (ANR), France.

Keywords: Thyroid - iodination - proteomics – toxicology

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Evaluation of synaptic proteins as cerebrospinal fluid stage biomarkers for Alzheimer's Disease

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A biomarker capable of detecting synapse loss, which occurs early in Alzheimer's disease (AD) pathophysiology, would greatly assist in preclinical diagnosis, when treatment would most likely be effective. The objective of this study was to identify and quantify synaptic protein levels in cerebrospinal fluid (CSF) in the search for preclinical biomarkers of synaptic loss. Shotgun LC-MS/MS was performed on pooled CSF samples from cognitively healthy subjects and AD patients. A total of 2,731 CSF proteins were identified by at least one peptide. The CSF proteome was filtered for proteins that i) have a known synaptic function and ii) have been reported as co-enriched with PSD, synaptic vesicle or whole synaptosome proteins in post-mortem tissue. 165 synaptic proteins detectable in the CSF were retrieved. A panel of 22 proteins was selected by manual curation. Synaptic expression of the panel was confirmed by Array Tomography (AT) microscopy and synaptosome enrichment of human cortical tissue. Quantification of up to 3 unlabeled peptides per protein was performed by targeted Selected Reaction Monitoring (SRM) in 2 independent clinical cohorts (n=192). Log2-foldchanges were calculated for each diagnostic stage versus cognitively healthy subjects using the MSstats package in R. ROC curves were generated to determine diagnostic accuracy. Compared with cognitively healthy subjects, the CSF levels of 7 synaptic proteins were reduced 0.7-0.8-fold in non-symptomatic preclinical subjects (all $p<0.03$), all proteins were increased 1.19-1.34-fold (all $p<0.01$) in subjects with mild cognitive impairment (MCI) and 6 proteins were increased 1.1-1.2-fold in dementia patients (all $p<0.03$). The best diagnostic accuracy (AUC) for preclinical subjects was 70% (CI 54-85%) and for MCI subjects was 74% (CI 62-85%). In conclusion, characterization of the CSF and synaptic proteomes followed by targeted SRM identified a panel of synaptic proteins that could be used to monitor synaptic damage in clinical for AD.

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Identification of proteomic biomarkers of arsenic toxicity and evaluation of dietary curcumin supplementation for mitigation

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Background: Arsenic is a highly carcinogenic environmental contaminant and arsenic toxicity (arsenicosis) is a major public health concern in many countries. Arsenicosis leads to clinical manifestations like melanosis, keratosis and cancer of varius organs in humans. Early diagnosis would help in better management of patients. To identify biomarkers of arsenicosis, we carried out plasma proteomic analysis of arsenic-exposed Labeo rohita and identified ApoA1, A2ML, Wap65 and Transferin as potential biomarkers (J Hazard Mater 336:71-80.). To identify which isoform of the gene ApoA1 better responds to arsenic toxicity, we carried out gene expression analysis of three ApoA1 variants viz. ApoA1-1, ApoA1-2a and ApoA1-2b and also investigated whether dietary supplementation of curcumin, a potential herbal drug/food supplement, has any ameliorative potential against arsenicosis.

Methods: Fishes were divided into seven groups; three groups were fed with basal diet; two groups were fed a low-dose of curcumin-supplemented-basal diet and two were fed high-dose of curcumin-supplemented-basal diet, for seven days prior to arsenic (Sodium meta arsenite:NaAsO2) exposure and this continued during the exposure period. One of the basal diet fed groups served as the control and the other two groups were exposed to 5- and 15-ppm arsenic, respectively. After the exposure period, fishes were sacrificed, liver tissues were taken out and gene expression analysis of ApoA1 variants was carried out.

Results: The ApoA1-1 and ApoA-2a variants were down-regulated in arsenic- exposed fishes and their expression was found to be same as in control in the curcumin-supplemented-diet-fed fishes. Expression of ApoA-2b variant was only found in arsenic-exposed fishes but not in the control and curcumin-supplemented-diet-fed fishes.

Conclusion: This study showed that ApoA1-2b responds well to arsenic toxicity and down-regulation of ApoA1-2b proves the efficacy of curcumin against arsenicosis, in a dose-dependent manner. ApoA1-2b appears to be a diagnostic marker of arsenicosis.

901

Proteome variation related to thyroid regulation by iodide

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Background:
Iodide is an essential element required for the synthesis of thyroid hormones (triiodothyronine (T3) and thyroxine (T4)). It is well established that thyroid hormone synthesis is regulated by TSH (Thyroid Stimulating Hormone) but other studies indicate that thyroid function is also controlled by circulating plasma iodide.

Circulating iodide is captured by thyroid. In case of high iodide intake, this leads to a thyroid regulation mechanism called Wolff-Chaikoff effect. Previous studies reported that iodide inhibits several steps of thyrocyte activity as iodide uptake (mediated by the sodium iodide symporter), iodine organification (mediated by thyroperoxidase) and thyroid hormones secretion.
Until now, the molecular mechanisms involved in this physiological response remain unknown.

Methods:
Rodents were exposed to different diet conditions to induce or not a Wolff-Chaikoff effect and study the mechanisms involved.
We combined free labelling (Shotgun) and labelling (Tandem Mass Tag10plex) proteomics approaches to study thyroid proteome variations. Peptide identification was performed using liquid chromatography coupled with an Orbitrap QExactive spectrometer.

Results:
Our strategy allowed us to identify global changes in thyroid proteomic profiles induced by iodide. Our results highlight putative pathways involved in this regulation.

Conclusion:
Our study showed that proteomics approach is an efficient strategy to provide new insights into thyroid function and its regulation by iodide.

This work was supported by the 'Commissariat General a l'Investissement' ('RSNR' program) and the 'Agence Nationale de la Recherche' (ANR), France.

Keywords
Thyroid, Wolff-Chaikoff, quantitative proteomics, iodide regulation

902

DigiWest based focused Proteomics of irradiated human embryonic stem cells and neuroepithelial cells

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Background:
3D-cell culture in-vitro test systems provide primary tissue like test conditions resulting in better predictions of drug effects in vivo as 2D-cell culture models. Long term effects of ionizing radiation on the developing brain of embryos cannot be measured directly. Therefore 3D-cell models based on human embryonic stem cells provide a valuable model system to measure long term effects of irradiation damage on brain development in cells surviving irradiation.
Methods:
Human embryonic stem cells (H9, cell line) were differentiated into neuroepithelial cells (NEPs) which were irradiated at day 9 during differentiation with X-rays at different doses (0 / 0.5 / 1 Gy). At day 11 and after differentiation into neurospheres (NS) at day 18 cell samples were collected and analyzed for differences in protein expression by DigiWest technology. Furthermore H9 stem cells were directly irradiated and analyzed at 24h and 48 h after irradiation by DigiWest. More than 446 antibodies were used to test for histones, cytoskeleton proteins and proteins and their phospho sites from major metabolic and signaling pathways.
Results:
222 antibodies tested showed valid signals in the DigiWest assays. Significant differences in protein expression were found for NEP and NS. Furthermore differences in protein concentrations were found for different irradiation doses and post-irradiation times. Feeder cells could be differentiated from H9 cells.
Conclusions:
DigiWest is a valuable tool to perform focused proteomics on tiny sample amounts from in-vitro test systems. Ionizing irradiation showed long term changes in protein expression and pathway activation in survivor cells

Keywords:
DigiWest / focused proteomics / embryonic stem cells / in-vitro test systems / irradiation damage

903

Evaluation of Mitra microsampling device for Multiple Reaction Monitoring based measurement of proteins in serum

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Background
The Mitra© microsampling device is a biological specimen collection method based on Volumetric Absorptive Microsampling technology (VAMS™). Mitra sampler tips consist of a hydrophilic porous material designed to take up a fixed volume (10µL) biological fluid by capillary action. As such Mitra samplers offer significant advantages for quantitative sampling of whole blood compared to the use of paper/dried blood spots (DBS). There is considerable interest in the use of DBS for measurement of proteins. Here, we evaluated the use of Mitra microsamplers for sampling serum and subsequent targeted protein measurement by multiple reaction monitoring (MRM).
Methods
Mitra microsamplers were dipped into a laboratory reference serum sample until they were loaded as recommended by the manufacturer (Neoteryx). The sampler tips were removed and placed into 96-well plates followed by tryptic digestion using a standard protocol. As controls, liquid serum samples were digested in parallel. Following proteolysis, 18 abundant serum proteins were measured by MRM using an Agilent 6490 QqQ mass spectrometer.
Results
Data from the MRM measurement of abundant serum peptides produced by tryptic digestion of serum in liquid form or dried on Mitra samplers produced similar individual peptide measurements. Interestingly, samples loaded onto Mitra microsamplers produced a 50% improvement in assay-to-assay reproducibility than liquid sampling.
Conclusion
Mitra microsampling devices provide a practical, user friendly quantitative serum sampling device for subsequent use in targeted protein MRM measurements. Their ease of use and increased level of precision compared to liquid sampling allows for more consistent sample preparation and reduces the opportunity for pre-analytical errors. The data obtained suggests that the potential use of Mitra devices for sampling, shipping and storing serum prior to measurement of serum protein biomarkers by MRM merits further detailed investigation. The significant advantages of this sample workflow will be described.
Keywords
Serum, MRM, protein biomarkers, sample preparation

904

Multiplexed MRM-based Protein Quantitation Using Two Different Stable Isotope Labeled Isotopologues Peptides for Calibration

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Background

Precise and robust quantitation of the endogenous plasma proteome is a requirement for fundamental and biomedical research as well as for clinical applications. Targeted detection of peptides in a bottom-up strategy is the preferred mass spectrometry-based quantitation approach when combined with stable isotope labeled peptides. However, when measuring protein in plasma, endogenous levels prevent the implementation of the best calibration strategies since no blank matrix is available. Consequently, several calibration strategies are employed. In our recently published study, these were compared to a new approach using two different stable isotope-labeled standard (SIS) peptide isotopologues for each peptide, enabling an external calibration curve and QC samples in human plasma without interference from endogenous peptides.This strategy improves the analytical performance of the assays and facilitates development and validation.

Methods

Calibration strategies and matrices were evaluated and compared (31 peptides, n=5, 3 QC levels), using three isotopomers (light, heavy, and double labeled). Single point measurement, standard addition, and reverse curve strategies were evaluated. Human plasma (also dimethylated plasma), chicken plasma, PBS+BSA, and PBS matrices were tested. Samples were denatured, reduced and alkylated, and tryptic digested before solid phase extraction. Reversed-phase separation was performed over 30-min gradient. The UHPLC system (Agilent 1290 Infinity) was coupled to an Agilent 6490 triple quadrupole in MRM mode.

Results

The calibration approach with two isotopologue peptides clearly outperformed single point measurements and reverse curve methodologies. We show how this strategy does not introduce accuracy bias in the measurement compared to reverse curves, and how method development and validation are streamlined.

Conclusions

We have shown our method is an improvement on calibration strategies that are currently used and it is now being validated for a panel of clinically relevant protein targets in biological samples.

Keywords

Peptide quantitation; MRM; stable isotope labeled peptides; calibration; assay validation

905

SDS vs urea: fast and sensitive non-mechanical conversion of tissues to peptides

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Background

Human tissues are valuable sources of molecular information for classification and diagnosis. Minimally invasive collection of small tissue sections is hereby important. However, very few generally applicable methods exist that are commonly employed. Here we report on the evaluation of straightforward and fast methods for proteome analysis of small tissues. We observe that high-sensitivity is afforded by SDS, and a wide-variety is obtained when using urea-based methods.

Methods
Cryo-sectioned fresh-frozen human uterus tissues were placed on glass slides. Proteins from the tissues were extracted using several extraction methods that varied in SDS, the amount of organic solvent (30% or 60% ACN) and duration and method of tryptic digestion (18 or 18+4 hours). Tissues were further analysed by µLC/ESI-MS/MS.

Results
We evaluated several methods on minute tissue sizes ranging from 0.2 to 2 mm2 tissue, at 10, 16 & 20 µm thicknesses. We report that methods with SDS result in less identified proteins overall, compared to ACN/urea only. However, SDS-extracted proteins showed higher concentrations across the samples, underscoring that SDS is important for protein solubilisation. However, urea-based methods showed that a wider variety of identified proteins could be found in all the tissue sizes.

Conclusions
Both, SDS- and urea-based buffers showed to be robust for protein screening and quantitation of minute tissue sections. Urea-based methods showed higher number of identified proteins, and the use of SDS revealed a higher amount of those proteins measured. As expected, an increase in the amount and thickness of tissue led to an increase in number and amount of identified proteins, but this increase was not linear.

906

Biopharmaceutical Characterization using Size Exclusion and Cation Exchange Chromatography and High Resolution Native Mass Spectrometry

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Thorough characterisation of therapeutic proteins is essential at all stages of development and manufacture . Monoclonal Antibodies (mAbs) are the fastest growing class of drugs due to their high specificity to the targets they are used against. Each mAb has several different variant forms due to multiple post translational modifications that can occur during production, purification and storage. These modifications can alter the charge distribution on the surface of the protein and can be characterised by charge variant analysis using ion exchange chromatography. All modification variants on the Mab require characterisation and control to ensure product quality and reproducibility as they could have an impact on efficacy or safety. Identification of structural variants is a critical challenge and mass spectrometry (MS) is an essential tool in the characterization of protein variants. However, ion exchange and size exclusion chromatography of proteins require high salt eluents which are incompatible with MS requiring that structural variants exposed by these techniques be collected off-line, then desalted before characterisation by MS. Here we describe novel direct on-line coupling of ion exchange to the MS instrument in the characterisation of mAb variants. The technique has a fast run time and greatly reduces analysis time and sample handling by avoiding fraction collection and separate desalting injections by reverse phase LC-MS. Chromatographic resolution of mAb charge variants using pH gradient elution with a novel volatile buffer preparation compares favourably with traditional salt elution. mAbs enter the Orbitrap MS in the native state with a reduced charge distribution and an elevated mass to charge ratio. Variants found using this direct on-line multi attribute coupling method include glycosylation, deamidation, oxidation and lysine truncated forms.

907

The integration of proteomic, imaging and clinical features into a classifier that risk-stratifies lung nodules

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Background:
Over 1.6 million lung nodules are detected annually in the US. Nodule management depends upon the physician's cancer risk assessment (CRA). Low-risk nodules are monitored by CT but many undergo unnecessary invasive procedures: biopsy and surgery. This research explores the laboratory and computational methods used to integrate proteomic, imaging and clinical risk features for improving the CRA so that unnecessary invasive procedures are reduced. The resulting diagnostic tool, Xpresys Lung (XL), is the first multiplexed mass spectrometry-based test used in clinical practice with government reimbursement approval.

Methods:
The development of XL has occurred over five studies spanning 1,548 samples (1,160 were from two prospective studies) with plasma collection on nodules 8-30mm in diameter. Imaging and clinical factors were also collected and multiple reaction monitoring mass spectrometry (MRM-MS) was used to measure the relative abundance of two plasma proteins (LG3BP and C163A). Over the course of the first four studies, an integrated classifier (XL) was developed that combined imaging features (nodule size, location, morphology), clinical features (age, smoking history) and proteomic features (the ratio LG3BP/C163A). The fifth study assessed the analytical and clinical performance of XL.

Results:
First, the clinical performance of XL on 178 intended use samples: sensitivity 97% (CI 82%-100%), specificity 44% (CI 36%-52%), NPV 98% (CI 92%-100%) in distinguishing benign nodules. Second, the analytical performance of XL had an inter-batch precision with CVs of 8.0%, 14.5% and 12.8% for LG3BP/C163A, LG3BP and C163A, respectively. Third, as in prior studies, the proteomic features were the most informative of all features.

Conclusions:
Proteomic features, measured by MRM-MS, can be integrated with imaging and clinical risk features to provide accurate risk stratification of nodules for clinical practice. Additionally, the use of a proteomic ratio significantly improves analytical precision.

Keywords: mass spectrometry, clinical proteomics, lung nodules, multiple reaction monitoring, lung cancer

908

Revealing the systemic disorders underlying environmental enteropathy using in vivo proteomics of SILAC-mice, and metabolomics

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Stable Isotope Labelling with Amino acids in Cell culture (SILAC) has become a standard tool in quantitative proteomics. However, it has several limitations, including being restricted to cell culture. As a powerful alternative, mice can be fed diets containing 13C6-substituted lysine to completely label their proteome. This enables mouse models of disease to be used to ask new, more medically relevant questions.

Environmental Enteropathy (EE) is an inflammatory disease of the small intestine. While typically being subclinical, it is a major cause of persistent malnutrition in children, especially in developing nations. However, the aetiology of EE still remains largely mysterious. Recently, our group developed a mouse model of EE. In this model, malnourished mice are orally exposed to a defined mixture of Bacteroidales and Escherichia coli bacteria, inducing growth stunting, intestinal permeability, and inflammation. Unexpectedly, liver from EE mice also showed significant fat deposits, which are indicative of metabolic dysfunction. This suggests that oral exposure to certain bacterial species precipitate metabolic disorders.

We conducted a multi-omics study of livers of EE mice. By combining quantitative proteomics of EE SILAC-mice with metabolomics data, we have begun to unravel the signalling pathways that underlie the systemic disorders that underlie EE. This will enable improved treatment of at-risk children in malnourished environments.

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Aggregation as a consequence of glycation: insight into the pathogenesis of arthritis.

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BACKGROUND: Advanced glycation end products (AGEs) as a result of Maillard reaction are currently at the heart of the pathogenesis of several diseases and hence are the objective of numerous investigations.
METHODS: The purpose of this study was to monitor and characterize the oligomeric aggregates and AGEs of human collagen on addition of glyoxal using ultraviolet, fluorescence, circular dichroism (CD) spectroscopy, docking studies, ITC, and microscopy.
RESULTS: Collagen was incubated for varying time periods up to 21 days with three different concentrations (5, 20, and 40 mM) of glyoxal. Collagen exists as molten globule at day 6, evident from native-like secondary structure, altered tryptophan, and high ANS fluorescence due to surface-exposed hydrophobic residues. Glycated collagen as AGEs and aggregates was observed at day 18 and 21, respectively. Formation of AGE and aggregates were confirmed by UV and fluorescence spectroscopy. The obtained AGEs were characterized with respect to the extent of side chain modifications (lysine and arginine) forming the Schiff base, the carboxymethyl lysine, and carbonyl content. Non-tryptophan fluorescence for AGEs was also monitored as the emission peak at 400 and 440 nm, respectively. SEM and TEM confirmed the oligomeric nature of aggregates. Glyoxal at 40 mM shows maximum alterations in protein structure followed by 20 and 5 mM concentration. In the present paper, we propose that a high concentration of glyoxal for a prolonged time results in the formation of harmful aggregates and AGEs.
DISCUSSION: In present times, many studies have targeted the characterization of aggregates of clinically significant proteins. Glycation of proteins has been an implication in long-term complications. Collagen is the most abundant protein in the human body.
Keywords: collagen, Advanced glycation end products, aggregates, microscopy

910

A New Database Search Scoring Method for Better Identification Performance of Endogenous Small Peptides

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Background
Although the MS-based proteomics approach was developed to detect and quantify the peptides in a highly sensitive and efficient manner. However, it is still inefficient to detect the endogenous signaling peptides, because the enzymes for hydrolyzing proproteins are mostly unknown, therefore all of the possible peptide cleavage ways need to be considered in the peptide identification using database search. This non-enzyme specific database search can result high false identification rate, thus reduce peptide identification sensitivity. Here we developed a new approach for better identification of the cellular peptide. The performance and the application of this approach was studied and discussed.

Methods
The peptide standard mixtures and cellular peptides extracted from Jurkat was analyzed by the LC-MS and the acquired spectra was processed for the detection of fragment peaks. The 1st to 10th peptide matching scores for each of the MS/MS spectrum were used to develop new peptide matching score.

Results
With the use of matched random/decoy sequences to estimate the false-positive discovery rate (FDR), the number and average score of estimated false-positive hits for non-enzyme specific (NES) search was 18.5% higher than the semi-tryptic peptide specific (sTPS) search. In this study, we developed a new scoring method named confidence scoring (CS), which considered the 1st to 10th matching scores of each spectrum. For a sample containing 286 know peptides, with the FDR<0.01, the number of the unique sequences discovered by Mascot and CS was 157 and 200. There were 152 peptides commonly identified in Mascot and CS. In contrast to the Mascot, CS identified 25% more peptides that was not identified by Mascot but the DS only missed 5 peptides identified in Mascot.

Conclusion
This method does not require complex computational steps and significantly improve the sensitivity for novel signaling peptide discovery.

Keywords
Signaling Peptides, Database Search, Mass Spectrometry

911

Proteomics for Rare Cancer: Lesson Learned from Sarcoma Biomarker Research

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Background: Rare cancers are identified as those with an incidence of less than 6 per 100,000 persons per year. There are approximately 200 malignancies, which are defined as rare cancers. Collectively, the rare cancers are not rare; they represent 22% of all cancer cases diagnosed each year in Europe, and 15% in Japan. There are many challenges in the clinical practice as well as the research in the rare cancers, because of their rarity. To name a few, a clinical trial is difficult to perform, because of the shortage of participants to the studies. As a consequence, the overall survival period is significantly shorter in the rare cancers than that of common cancers. The biomarkers will be a remedy for the rare cancers. The personalized medicine and clinical trials will be promoted by stratifying the patients according to the outcome expected by biomarker examinations.

Methods: This study focused on a typical rare cancer, sarcoma. Tumor tissues were examined by 2D-DIGE and mass spectrometry, and analyzed with clinical data. The patient-derived xenografts (PDXs) were developed for functional verification of biomarker proteins and the drug response study.

Results: We have developed biomarkers for differential diagnosis, or prediction of treatment outcome. We validated the clinical significance of our biomarkers in the multi-institutional studies. We found the proteome of PDX models was similar but not identical to that of their original tumors.

Conclusions: The clinical utility of our biomarkers is under validation in the prospective clinical study. The understanding of proteomic changes during the process of model development is required to make the best use of PDX models. Considering unique and divers genetic alterations such as chromosomal translocation, the accuracy of protein identification should be improved. In this sense, proteogenomics is worth challenging in the rare cancers.

Keywords: rare cancer, sarcoma, biomarker, proteogenomics

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Impact of AssayMap Bravo Liquid handling Platform on high throughput protein digestion for quantitative proteomics

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Background
Multiple reaction monitoring (MRM) is a highly effective tool for quantitative high throughput proteomic studies. Proteolytic digestion is an important pre-analytical step in MRM based proteomics and represents a major source of potential variation. Reproducibility can be compromised during tryptic digestion and it is acknowledged that more robust workflows are required to support large scale studies. Thus, automated approaches to undertaking proteolysis have emerged as attractive alternatives to manual approaches. Here we assessed a semi-automated approach using the AssayMap Bravo (Agilent) consisting of a positive displacement multichannel pipetting device (AssayMap) and associated liquid handling platform (Bravo).
Methods
A reference serum sample was reduced, alkylated and digested with trypsin using a manual and automated approach (AssayMap Bravo). Following proteolysis, peptides were analysed using an in-house MRM assay measuring 18 high abundant serum proteins. Peptides were separated on an analytical column (Zorbax Eclipse plus C18, rapid resolution) prior to analysis on a QqQ mass spectrometer (Agilent 6490). Subsequently data was imported to Skyline software (Version 3.6) where peptide peak areas were integrated.
Results
Comparative analysis revealed a 40% improvement in peptide peak area %CVs when digestions were performed using the Bravo workstation vs manual approach. Interestingly, data reproducibility was influenced by sample location on the 96 well plates. Thus, %CVs were 23% higher for the 36 outer wells compared to the 60 inner wells with the four 'corner' wells producing the highest variability.
Conclusions: The AssayMap Bravo is a flexible and user friendly platform for reproducible serum protein digestions in moderate to high throughput that can be used for subsequent quantitative MRM proteomics.

Keywords:
AssayMap Bravo, serum, protein digestion, MRM

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Use of Andrew liquid handling robot for protein digestion of serum for quantitative proteomic workflows

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Background
Reproducible protein digestions are essential for quantitative proteomics. Reproducibility may be compromised during reduction, alkylation and proteolysis and it is widely recognised that more robust workflows are essential for large scale studies. Thus automated approaches to proteolysis are attractive alternatives to the manual approach. Here we evaluated a liquid handling platform, Andrew (from the Andrew Alliance), to undertake semi-automated serum protein digestion. Andrew uses standard Gilson pipettes (P2 to P1000) and a 9-unit deck (domino) comprising of pipette tip boxes, reagent reservoirs and waste containers with each unit being identified by using 2D bar code labels and built in camera.

Method
A reference serum sample was reduced, alkylated and digested with trypsin following a manual and automated approach (Andrew). Following proteolysis, peptides were analysed using an in-house MRM assay measuring 18 high abundant serum proteins. Peptides were separated using an analytical column (Zorbax Eclipse plus C18, rapid resolution) before analysis on a QqQ mass spectrometer (Agilent 6490). Subsequently data was imported to Skyline software (Version 3.6) where peptide peak areas were integrated.

Results
Comparative analysis revealed an 19% improvement in peptide peak area % CVs when digestions were performed using Andrew vs. manually. Interestingly, sample location on a 96 well plate influenced data reproducibility. Hence, % CVs were 19% higher for the 36 outer wells compared to the 60 inner wells with the four 'corner' wells generating the highest variability.

Conclusion
Andrew is a flexible device that can be easily integrated into a semi-automated workflow for reproducible serum protein digestions in moderate to high throughput. Digestions performed with Andrew can be used for subsequent quantitative MRM proteomics.

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Vascular Niche E-Selectin Plays A Key Role In Leukemia Chemo-resistance

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Background
Acute myeloid leukaemia (AML) is a haematopoietic malignancy which resists cancer therapy partly by hijacking the bone marrow (BM) niche. The BM niche is a specialised microenvironment which supports haematopoietic stem cells. We found vascular adhesion molecule E-selectin to be a key niche component mediating AML chemoresistance in vivo. Here, we investigated the mechanisms involved in E-selectin-mediated chemoresistance.

Methods
Using an in vitro cell culture model we showed AML cells in contact with E-selectin had a significantly increased survival to chemotherapy. Next we used mass spectrometry proteomics to characterised the receptors used by AML cells to interact with E-selectin and potentially mediating chemoresistance. Using in-vivo knock-out mice models, we showed that AML cells lacking these receptors no longer demonstrated E-selectin-mediated chemoresistance in our in vitro assay.

Results
AML cells in contact with E-selectin had a significantly increased survival to chemotherapy in vitro, which could be reversed by an E-selectin function blocking antibody. Putative receptors to E-Selectin were identified by mass spectrometry, including canonical E-selectin receptors CD44 and PSGL-1. We showed that AML cells lacking identified receptors no longer demonstrated E-selectin-mediated chemoresistance in our in vitro assay. These results suggest that CD44 and/or PSGL-1 may be the receptors involved in niche-mediated AML chemoresistance.

Conclusions
This in vitro study confirms our earlier in vivo data, and together these findings may lead to new adjuvant niche-based strategies to improve the efficiency of current AML treatments.

Keywords
Acute myeloid leukaemia (AML)
Chemoresistance
E-selectin receptors

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Omics investigation of FDG-PET based heterogeneity in solid tumors

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Phenotypic and functional heterogeneity arise among solid tumors as a consequence of somatic aberrations, interstitial environment differences and reversible changes in cell properties. The heterogeneity observed within tumors and the myriad of genome instability processes that shape tumor evolution have important clinical implications and may reflect the mismatch between cost and benefit of some anticancer therapies. As these genetic alterations translate to proteomic/metabolomic level, a holistic view of tumors heterogeneity is needed. Despite the development of analytical quantitative methods in molecular system biology, several studies failed to capture the biological signature of inter/intra-tumor heterogeneity. Accordingly, accurate delineation of heterogeneous tumors within solid tissue is a crucial step to reach a systematic depiction. Therefore, FDG-PET imaging offers a valuable tool in clinical diagnosis, staging, and cancer monitoring, as glucose uptake is an indicator of tumor heterogeneity and plasticity. FDG-PET imaging was performed on MMTV-PyMT breast cancer mouse model with highly heterogeneous tumors. Subsequently, imaging data was used to delineate 131 ROIs with differential glucose uptake in 24 mice. The ROIs were isolated with an in-house designed image-guided-milling machine. Using a multiplex extraction method, we isolated metabolites and proteins. Thus, samples were analyzed using shot-gun label-free-quantitative proteomics (Orbitrap Fusion Tribrid), targeted LC-MS metabolomics (Biocrates IDQ180 AB-SCIEX-QTRAP-6500) and untargeted 1H-NMR spectroscopy (Bruker-600MHz-Avance III).
In-depth data analysis of high versus low uptake regions showed a clear separation based on proteins and metabolites abundance using PCA analysis. Distinctly, we detected alterations of specific tumor driver proteins, transcription factors, and interferon induced proteins. Furthermore, metabolomic analysis showed discrepancies in amino-acids, polyamines, and phosphatidylcholines. Altogether, our analysis shows a specific proteomic and metabolomic profile defined by the FDG-PET uptake of tumor regions within the oncogenic tissue. Thus, our analysis is the first attempt to accurately characterize tumor heterogeneity using an imaging-guided approach in tandem with proteomics/metabolomics investigation.

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Quantifying and localizing the mitochondrial proteome using SWATH-MS

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Mitochondria are dynamic organelles essential to a range of metabolic processes. Despite our increased knowledge of mitochondrial functions, we do not yet completely understand the mitochondria's variable proteomic composition and regulation—even for core functions such as OXPHOS. Recently, SWATH-MS enables accurate quantification of ~4000 proteins across large number of samples and thus presents the possibility to rapidly and comprehensively quantify proteins under multiple conditions.
Here, we demonstrated the additional possibility of SWATH-MS to determine cellular localization and redistribution of proteins. We have used SWATH mass spectrometry to quantify 3648 proteins across 114 proteomes collected from genetically-diverse BXD mice in two fractions (mitochondria or total cell) and five tissues: liver, quadriceps, heart, brain, and brown adipose tissue (BAT). Protein expression showed stronger variation across tissues than across genetic backgrounds, and proteins broadly correlated with transcriptome measurements in the same individuals. Interestingly, we quantified similar number of mitochondrial proteins in the two fractions, indicating that mitochondrial enrichment can be unnecessary for mitochondrial proteomics study using SWATH-MS. By comparing the two fractions, we observed several dozen proteins as mitochondrial, which were not reported in literature, of which three were validated in mitochondria (MTAP, SOAT2, and IMPDH2), and one in the mitochondria-associated membrane (ABCC6). While broadly correlated, proteins and transcripts had divergent regulation for certain key functional pathways, including the electron transport chain and adaptive thermogenesis. These findings demonstrate that SWATH-MS proteomics provides a basis for generating reliable, quantitative, and comprehensive proteomic maps of protein localization across cell and tissue types, and across cellular fractions.

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Considerations for selecting the optimal stationary phases for proteomic trap-and-elute nanochromatography

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Considerations for selecting the optimal stationary phases for proteomic trap-and-elute nanochromatography

Background
NanoLC/MS is a powerful tool to monitor low abundance peptides in complex proteolytic samples. The low flow rates used with small diameter columns enhance electrospray efficiency, and thus the analysis sensitivity. The increased MS sensitivity, however, is often compromised by poor chromatography because nanocolumns cannot accommodate large injection volumes. The trap-and-elute method is a perfect solution to this problem. It allows a comparably large volume of sample to be injected without the risk of sacrificing peak shape or fouling the column. An optimized trapping condition further increases the method robustness and extends the column lifetime by preventing impurities in samples, such as salts and strongly retained compounds, from entering the nanocolumn.

Methods
We tested several commercially available traps and nanocolumns for their retentivity, trapping efficiency, and chromatographic performance in a typical proteomic separation.

Results
The ability to use different trap and nanocolumn stationary phases provides great flexibility in developing the analysis methods. The combination should be carefully selected based on the target peptides and the retention properties of the stationary phases. Incompatible pairs cause significant peak broadening, which in turn results in the loss of detection sensitivity and analysis quality.

Conclusions
There are three important selection considerations in optimizing a trap-and-elute nanoLC/MS method: 1) the absolute retentivity of the trap and the nanocolumn, 2) the retentivity difference between the trap and the nanocolumn, and 3) the dispersion contribution from the trap.

Keywords
Nanoflow chromatography, trap-and-elute, peptide separation, LC/MS

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A novel nanoflow LCMS limited sample proteomics approach using micro pillar array columns (µPAC™)

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A novel nanoflow LCMS limited sample proteomics approach using micro pillar array columns (µPAC™)
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Bottom-up proteomics using 50 to 100 µm C18 packed capillaries coupled to high resolution mass spectrometers is now established as the common workflow to analyze protein samples from tissues, body fluids or cell lysates. Typically, micrograms of samples are separated in 30 min to 4 hour nano LC gradients resulting in the identification of 4000 to 5000 protein groups. However, ease-of-use and reproducibility of nanoflow LCMS using packed capillaries does not yet allow novice and routine use.
PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is a unique and novel approach to a chromatographic support structure and builds upon micromachining chromatographic separation beds into silicon. The low 'on-column' dispersion obtained by the resulting perfect order separation bed virtually eliminates axial peak dispersion, resulting in higher column plate numbers with sharper peaks and higher concentration of compounds. The freestanding nature of the pillars also leads to much lower backpressure allowing the use of very long columns. These exceptional properties result in excellent chromatographic performance with high-resolution and high sensitivity.
Here, we are presenting data on testing this novel approach to a nanoflow column in a bottom-up proteomics workflow. Coupling a 2 m long µPAC™ column via a nanoflex source to a Thermo Fisher nLC1200 pump and a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer, we used standard proteomics separation and electrospray conditions, e.g. a flow rate of 300 nl/min with a 3 to 32% gradient of 0.1% formic acid to acetonitrile/0.1 % formic acid in 2 and 4 hours, an ESI voltage of 1900 V with a 10 µm silica emitter. The Fusion™ Lumos™ Orbitrap™ mass spectrometer was operated at a resolution of 120000. Injecting a dilution series of 1 µg, 100 ng and 10 ng, we observed in triplicate runs at the highest concentration 5400 protein groups in a 4 hour gradient run. Interestingly, when injecting only 10 ng HeLa cell digest, corresponding to the content of 50 cells, we still see over 3000 protein groups. Thus, this workflow using µPAC™ columns and a Fusion™ Lumos™ MS is suitable to proteomics experiments where the sample amount is very limited to a small number of cells and therefore opens up a new tool for biologists.

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Quantitative DUB interactomics to investigate the biology of DUBs

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Quantitative DUB interactomics to investigate the biology of DUBs

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Deubiquitinases (DUBs) are proteases that cleave ubiquitin off substrates, and thereby regulate ubiquitin signalling. DUBs have been implicated in a number of diseases including cancer and neurodegeneration. Despite their obvious importance, the biological roles of many DUBs remain to be defined. This is in part because the identity of the cellular substrates of most DUBs is not defined. Identifying the substrates of DUBs is not feasible using standard biochemical approaches owing to the transient nature of DUB-substrate interactions. To systematically investigate DUB function, we used proximity-based biotin labelling (BioID), a unique strategy that has the capability to capture even transient and weak affinity protein-protein interactions when combined with high-resolution mass spectrometry. Using combined MaxQuant and Significance Analysis of INTeractome (SAINTexpress) analysis, we determined the interactomes of 35 different human DUBs. Systematic analyses revealed unique subsets of proteins for each DUB profiled. In addition to recapitulating some of the previously reported interactions of DUBs, our approach reveals novel interactors. Importantly, this method proves to be especially powerful when studying organelle- and subcellular-specific interactions, which are challenging to study by conventional approaches. Further, we find many DUBs to interact with E3 ligases, suggesting a close inter-play between the ubiquitination and deubiquitination machinery. In summary, this method is a useful approach for the identification and study of DUB signaling networks.
Keywords:
Deubiquitinases; protein-protein interactions; system biology; cell biology

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Kojak: pipeline developments and new features for the analysis of chemically cross-linked proteins

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Title
Kojak: pipeline developments and new features for the analysis of chemically cross-linked proteins

Presentation Type:
Oral or Poster

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Abstract:
Background
Kojak is a versatile database search algorithm for the identification of cross-linked peptides from shotgun MS of chemically cross-linked proteins. Since its introduction, many new features have been added to Kojak that increase its utility and facilitate integration into larger data analysis pipelines, Key examples include virtually unlimited cross-linker customization, compatibility with ETD generated spectra, and results reporting in standardized open formats. These features enabled rapid integration of Kojak into larger data analysis pipelines that include the Trans-Proteomic Pipeline and the ProXL Protein Cross-linking Database.
Results
Using these advanced pipelines incorporating Kojak, users can easily expand their crosslinking analyses to include multi-file batching, access to additional results validation tools, and graphical visualization of results.
Keywords
Bioinformatics, crosslinking, Kojak, Trans-Proteomic Pipeline, TPP, ProXL