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Abstract Book

Abstract Topics

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P001

Proteome Integral Solubility Alteration assay in zebrafish embryo for exploring exposure to bioactive compounds including pollutants and chemicals mixtures

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Topic: Activity and Chemical Proteomics

The One Health initiative connects human and animal health with their shared environment. The development of novel methodology to unbiased identification of mechanisms of action of chemicals and chemicals mixtures would address one of today´s challenges of environmental and human toxicology. Zebrafish embryos offer a unique opportunity to explore the proteome integral solubility alteration (PISA) assay to study the proteins that increase or decrease their solubility as protein targets of the bioactive compounds in an organism at an developmental stage. We presented here 4 different applications of the PISA method in zebrafish embryo with different types of compounds of environmental and health concern: i) endocrine disrupting chemical, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to outline the pathways compromised under exposure; ii) complex chemical mixtures composed by TCDD + alpha-endosulfan + bisphenol A (BPA) previously studied in vitro cells lines to evaluate single chemical versus mixture; iii) novel compounds from blue-green circular economy to map the organismal response, and a iv) novel drug directed to anti-cancer activity in lymphoma models to discharge side-effects at non-target organs. Our results demonstrated the unique opportunities of PISA assay in zebrafish for toxicology. We remark some differences to its application for drug target deconvolution. First, we evaluate both targets arising from an increase in solubility and in those decreasing, as both would be involved in modulation the cellular functions. Second, the method could be applicable to bioactive compound before chemical characterization or a chemical mixture of unknown stoichiometry. Third, the results from zebrafish embryo could provide a first glimpse of unspecific interactions with proteins in non-target tissues. The introduction of PISA assay in toxi- and ecotoxicoproteomics will offer high-resolution and throughput in support with the 3R, and 3M principles.

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P002

Ultrasensitive, multiplexed enzyme activity profiling with soluble activity-dependent proximity ligation

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Topic: Activity and Chemical Proteomics

Measurement of protein activity, relative to abundance alone, offers a more accurate depiction of biological state. Detection and quantification of family-wide probe engagement generally requires LC-MS/MS or gel-based detection methods, which suffer from low resolution, significant input proteome requirements, laborious sample preparation, and expensive equipment.
To more readily enable translational applications, we developed a novel gel- and MS-free chemical proteomic platform, soluble activity-dependent proximity ligation (sADPL) for ultrasensitive and multiplexed quantification of active, endogenous proteins. sADPL integrates family-wide probe labeling with oligonucleotide barcoded antibody for proximity ligation and amplification, allowing for specific real-time quantitative PCR (qPCR) readout. In this manner, sADPL is implemented to “write” and “read” biomarker activities through barcoded amplicons quantification. sADPL demonstrated a linear dynamic range 3 to 4 orders of magnitude over proteome concentration and at leat 6 orders of magnitude lower then western blot analysis, which could detect active protein in pictogram quantities of whole proteome. We applied sADPL in two translational applications. First, this approach enabled simultaneously quantify small molecule inhibitor target engagement as well as occupancy of multiple “off-target” proteins in blood-derived peripheral blood mononuclear cells (PBMCs) after administrating the inhibitor in vivo, which suggested this method could be a general solution to the challenge of monitoring drug-target interaction profiles from patient-derived samples. Second, the method was used to quantify a panel of active enzyme biomarkers in biobanked, patient-derived ovarian cancer tissues, which revealed the reverse correlation between some enzyme activities with patients’ outcome. This result indicated the potential for discovering a new biomarker for ovarian cancer. Combined, the results are a compelling demonstration of the utility of this new method for diverse basic and translational proteomic profiling applications.

P003

The interaction between SSV1 transcriptional regulator F55 and RadA host protein is the molecular sensor of the UV-induced DNA damage

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Topic: Activity and Chemical Proteomics

Sulfolobus spindle-shaped virus 1 is the only UV-inducible member of the virus family Fuselloviridae. Originally isolated from Saccharolobus shibatae B12, it can also infect Saccharolobus solfataricus. Like the CI repressor of the bacteriophage λ, the SSV1-encoded F55 transcription repressor acts as a key regulator for the maintenance of the SSV1 carrier state. In particular, F55 binds to tandem repeat sequences located within the promoters of the early and UV-inducible transcripts. Upon exposure to UV light, a temporally coordinated pattern of gene expression is triggered. In the case of the better characterized bacteriophage λ, the switch from lysogenic to lytic development is regulated by a crosstalk between the virus encoded CI repressor and the host RecA, which regulates also the SOS response. For SSV1, instead, the regulatory mechanisms governing the switch from the carrier to the induced state have not been completely unravelled. Therefore, in this study we have applied an integrated biochemical approach based on a variant of the EMSA assay coupled to mass spectrometry analyses to identify the proteins associated with F55 when bound its specific DNA promoter sequences. Here, we show that the archaeal molecular components (F55 and RadA), which sense the host DNA damage, are functional homologs of λ (CI) and Escherichia coli (RecA) system.

P004

A robust and flexible approach for quantifying protein turnover rates across an entire proteome

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Topic: Bioinformatics and Statistics

Introduction
Cellular proteins are in a constant state of flux, continually being synthesized and degraded, and the rate of protein turnover is the coordinated action between protein synthesis and degradation. The ubiquitin-proteasome system (UPS) is one of the major processes that controls protein degradation and dysregulation of the proteasome can lead to many human diseases such as cancer. Protein turnover rates can be determined by pulse SILAC and while this approach has been used in numerous studies, there exists a need to develop software to analyze pulse SILAC data for determining protein turnover rates.
Methods
Human foreskin fibroblasts were cultured in light SILAC media and switched to media containing heavy SILAC for varying times. Cell lysates were prepared and analyzed by high resolution mass spectrometry. Protein turnover rate was modeled using the heavy fraction abundance as a function of time with a non-linear model. Estimates of protein half life for each protein was estimated by a novel algorithm described in the results.
Results
Here, we demonstrate a robust model for estimating protein half lives from pulse SILAC data that can fit data generated via DDA, PRM, or DIA, and is flexible to the number of timepoints and biological replicates included in the pulse experiment. Our approach assumes that peptides originating from a given protein (group) will have the same turnover profile and fits the fractional abundance of the peptides to an exponential decay model. We bootstrap across the peptides, timepoints, and, when present, biological replicates to estimate the protein half life mean and standard deviation. We explore the robustness of our model through simulations of number of timepoints and number of peptides per protein.
Conclusions
Here, we demonstrate a robust method for fitting protein turnover rates and compare across a variety of biological conditions.

P005

CoronaMassKB: an open-access platform for sharing of mass spectrometry data and reanalyses from SARS-CoV-2 and related species

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Topic: Bioinformatics and Statistics

Introduction: Despite the pressing need for detailed proteomics characterization of SARS-CoV-2/COVID19 biology, mass spectrometry (MS) analyses of these samples typically identify less than one third of all acquired spectra and reveal little about the diversity and quantitative variation of post-translational modifications (almost all datasets are shared with no search or quantitative results). CoronaMassKB addresses this need as an open community resource designed for the rapid exchange of MS data and extensive (re)analysis results, supporting the global community of scientists working towards understanding the biology of SARS-CoV-2/COVID19.

Methods: Deep reanalyses of millions of spectra from public datasets were integrated into an open knowledge base designed to facilitate reutilization for design and analysis of new experiments. Deep reanalysis of public datasets was conducted using the Maestro workflow, including MSPLIT spectral library search, MSGF+ database search and MODa and spectral networks open-modification search for reliable detection of unexpected modifications.

Results: CoronaMassKB is currently based on reanalysis of >7 million spectra from SARS-CoV-2 datasets and >20 million spectra from related viruses (including SARS-Cov, MERS and H1N1) in public mass spectrometry data. Our reanalysis more than doubled the spectrum identification rate and revealed an unprecedented level of diversity with hundreds of different modification types. Searching for modified peptides nearly doubled the number of peptide identifications and revealed >550 SARS-CoV-2 hypermodified peptides with 10+ distinct combinations of modifications, with >100 unique modification variants detected for a single peptide sequence.

Conclusions: Thousands of modified peptide variants were identified to 25 SARS-CoV-2 protein products, with thousands more also identified for related viruses. Over 7,000 host proteins were also detected across lysate profiles, infection time points and affinity-purification enrichment of viral interactors. Among tens of thousands of peptides mapped to host proteins, 339 mapped to regulatory regions of 92 drug-target proteins assessed to be interactors of SARS-CoV-2 proteins.

P006

How the UniProt Proteins API can facilitate the analysis of proteomics data.

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Topic: Bioinformatics and Statistics

Introduction
The analysis of proteomics data is inherently reliant on high-quality protein sequence databases. UniProt is a comprehensive, expert-led, publicly available database of protein sequence, function and variation information. It currently holds over 18,000 reference proteomes, that are constantly updated and reviewed based on collaborations with a variety of sources such as Ensembl, RefSeq, ENA and proteomics repositories such as ProteomicsDB, PRIDE, Peptide Atlas and MaxQB.

Methods
To facilitate searching of proteomics data, reference proteomes and associated annotations can be queried programmatically using the UniProt Proteins API which allows researchers to access and download UniProt data alongside large-scale genomic, proteomics and variation data. Data is available for download and querying in a range of formats; including XML, FASTA and PEFF.

Results and conclusions
This API case study illustrates the functionality and query interface that allows users access to the full UniProt database without needing in-depth knowledge of programmatic languages. The UniProt Proteins API facilitates the return and download of large-scale biological data to enable data integration and analysis of a far broader dataset, allowing users to gain a greater understanding of the biological processes they are investigating. This case study includes examples of how the UniProt Proteins API can be queried using results from proteomics datasets to return protein sequence data including isoform sequences, protein-protein interaction data, associated disease annotation, and protein-altering variant sites along with associated genomic coordinates.

All data are freely accessible from www.uniprot.org
The UniProt Proteins API is available at; http://www.ebi.ac.uk/proteins/api/doc

P007

New label-free methods for protein relative quantification applied to the investigation of animal model of Huntington Disease

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Topic: Bioinformatics and Statistics

Spectral Counts approaches (SpCs) are largely employed for the comparison of protein expression profiles in label-free (LF) differential proteomics applications. Similarly, to other comparative methods, also SpCs based approaches require a normalization procedure before Fold Changes (FC) calculation. Here, we propose new Complexity Based Normalization (CBN) methods that introduced a variable adjustment factor (f), related to the complexity of the sample, both in terms of total number of identified proteins (CBN(P)) and as total number of spectral counts (CBN(S)). Both these new methods were compared with the Normalized Spectral Abundance Factor (NSAF) and the Spectral Counts log Ratio (Rsc), by using standard protein mixtures. Finally, to test the robustness and the effectiveness of the CBNs methods, they were employed for the comparative analysis of cortical protein extract from zQ175 mouse brains, model of Huntington Disease (HD), and control animals. LF data were also validated by western blot and MRM based experiments. On standard mixtures, both CBN methods showed an excellent behavior in terms of reproducibility and coefficients of variation (CVs) in comparison to the other SpCs approaches. Overall, the CBN(P) method was demonstrated to be the most reliable and sensitive in detecting small differences in protein amounts when applied to biological samples.

P008

HaDeX: analysis of data from hydrogen-deuterium exchange mass spectrometry experiments

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Topic: Bioinformatics and Statistics

Hydrogen-deuterium mass spectrometry (HDX-MS) is an analytical tool for monitoring dynamics and interactions of proteins. In the glaring opposite to crystallography-based methods, HDX-MS allows a unique insight into the dynamics of the protein structure. Such data is larger and more complicated than static structure, thus requires a dedicated software suite. However, the majority of existing tools do not cover a satisfying analytic workflow, especially on the level of data presentation and proper statistical interpretation. We propose HaDeX (10.1093/bioinformatics/btaa587), a novel tool for processing, analysis, and visualization of output data from existing search engines used in HDX-MS experiments. HaDeX features functions supporting the whole analytical process, including preliminary data exploration, ISO-based uncertainty, quality control, and generation of customizable publication-quality figures. Advanced reporting functions support the reproducibility of the analysis. HaDeX is available primarily as a web-server (http://mslab-ibb.pl/shiny/HaDeX/), but his all functionalities are also accessible as the R package (https://cran.r-project.org/package=HaDeX) and standalone software.

P009

Boosting the number of peptide identifications and their confidence using INFERYS Rescoring

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Topic: Bioinformatics and Statistics

Introduction
Most database search algorithms compare experimental fragmentation spectra of peptides with lists of theoretical fragment masses corresponding to peptides from an in silico digest of a protein database to calculate similarity measures, while largely disregarding the intensity dimension. Automatically matching peptides to spectra in this way will yield false identifications of low-quality spectra or misrepresent their confidence. The standard method to control for erroneous matching of such spectra is the target-decoy approach that estimates the False Discovery Rate (FDR) in bottom-up proteomics experiments. Machine learning methods such as Percolator are commonly used to separate incorrect from correct matches, but their performance heavily depends on the calculated scores. Here, we show how intensity-based scores successfully circumvent common issues and challenges in peptide identification.
Methods
The described method has been implemented in Proteome Discoverer 2.5. In brief, INFERYS is used to predict fragment ion intensities of candidate peptides that were matched to spectra by SequestHT. Intensity-based similarities and distances are calculated, added to the search engine output and passed to Percolator. These additional features are orthogonal to traditional scores enabling Percolator to better distinguish between incorrect and correct matches, which significantly increases the confidence in peptide identifications.
Results
The power of rescoring is fully exploited when used for the most difficult searches (no enzyme searches), when the search space is huge and when peptides with very similar properties are expected. Analyzing an HLA Class 1 peptide data set with INFERYS Rescoring yielded an increase of identifications of 59% on PSM, 55% on peptide and 34% on protein level. Additionally, INFERYS Rescoring greatly improves the separation of target and decoy PSM scores.
Conclusion
INFERYS Rescoring boosts PSM, peptide and protein identification rates in classical tryptic datasets and improves new applications like immunopeptidomics, where 55% more peptides are identified.

P010

The PSI Universal Spectrum Identifier (USI)

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Topic: Bioinformatics and Statistics

Introduction: The Human Proteome Organization (HUPO) Proteomics Standards Initiative (PSI) is developing the Universal Spectrum Identifier (USI) standard. The USI provides a standardized format for referring to each publicly released spectrum from a dataset or from a spectral library. The USI enables exchange of important mass spectral evidence both in publications and in software implementations, to better apply FAIR (Findable, Accessible, Interoperable, Reusable) principles for mass spectra.

Methods: Through a community effort during PSI workshops, remote discussions, and shared on-line documents we have developed a draft method of encoding a multi-part key that is effectively a virtual file path to each publicly released spectrum.

Results: The draft specification is available for inspection and feedback at the PSI web site. It has been submitted to the PSI Document Process and is currently under formal review. Community feedback is actively sought prior to final ratification by the PSI. The USI has already been implemented by several repositories including PRIDE, MassIVE, jPOST, and PeptideAtlas. These implementations take the form of interactive web pages into which USIs can be pasted and the corresponding spectra viewed, as well as OpenAPI-based web services that enable transmission of spectra based on USIs.

Conclusions: The USI will enable greater ease in communicating the spectra and interpretations of those spectra that are crucial as supporting evidence of scientific conclusions. Further information, implementations, and examples are available at http://psidev.info/usi.

P011

BIONDA: A free database for a fast information on published biomarkers

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Topic: Bioinformatics and Statistics

Introduction: Gene and protein variations or expression patterns are often directly and specifically involved in human diseases. They are extensively researched with continuously enhanced omics technologies and used for clinical test assays or as potential drug targets. Consequently, there is a dramatically increasing number of biomarker-related scientific articles and it becomes unfeasible for researchers to know all of them. Moreover, access to structured information on biomarker-disease relationships is often restricted, limited to particular types of markers or diseases, outdated and/or user-unfriendly in the existing online databases.
Methods: To address these issues we implemented the novel biomarker database BIONDA. The relational database system is accessible via a web application where biomarker-disease relations can be queried.
Results: BIONDA provides structured information on all biomarker candidates published in PubMed articles. There is no limitation to any kind of diseases. To this end, PubMed article abstracts and renowned databases such as UniProt and Human Disease Ontology are used as sources for BIONDA's database entries. These are acquired automatically and updated regularly using text mining methods. BIONDA is available freely via a user-friendly web interface. As a specific characteristic, BIONDA's database entries are rated by a scoring approach estimating biomarker reliability.
Conclusion: Thus, BIONDA is a valuable knowledge resource for biomedical research.

P012

mzTab-M: A Data Standard for Sharing Quantitative Results in Mass Spectrometry Metabolomics - 2020 Update

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Topic: Bioinformatics and Statistics

Introduction: Working within the consortia of the Metabolomics Standards Initiative, Proteomics Standards Initiative, and the Metabolomics Society, we published mzTab-M in 2019 [1] as a common result format for reporting MS based small molecules analytical approaches. The format captures final quantification values, the underlying proof in terms of features measured directly from MS, as well as different approaches used to identify molecules (e.g. MS/MS, ...).

Methods: mzTab-M is a tab-separated, tabular text format backed by a textual specification linked to a JSON schema enriched by controlled vocabularies (CVs). mzTab-M supports both automated validation, and semantic validation. Furthermore, ambiguity in the identification of small molecules can be communicated clearly, as well as allowing to link back features and quantified summary data to their originating MS ions and scans.

Results: Since its initial publication in 2019, mzTab-M has been supported by a number of metabolomics/lipidomics software and repositories that provide importing and exporting functionalities. The Lipid Data Analyzer 2 fully supported mzTab-M, later on followed by GNPS, MS-Dial, and MetaboAnalyst. As of August 2020, implementation for im- and export for MzMine 3 is in progress, as part of a Google Summer of Code assignment. Updates and improvements of the R and Python implementations are in progress and will be finished by the end of 2020, simplifying integration with XCMS. Implementation of export functionality within Skyline is planned for 2021.

Conclusions: mzTab-M has seen wide adoption after its initial publication. So far, the format specifications have proven its flexibility and robustness. Changes pertained only to clarification of definitions and terminology. We look forward to further feedback from implementers and users of both the format and its implementations to maintain and adapt the format to further use-cases.

[1] Hoffmann, N., et al. 2019 Anal Chem 91; 3302–3310

P013

Accelerating the speed of ion library generation using fast microflow DDA and cloud processing

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Topic: Bioinformatics and Statistics

As DIA for quantitative proteomics continues to be used more broadly across studies, the ability to rapidly generate large ion libraries on selected samples is key. MicroflowLC enhances both the robustness and speed of this process. Here the use of very fast gradients for DDA acquisition combined with data processing in a cloud-based processing solution was explored to accelerate ion library generation.
Methods: 40 high pH fractions of both plasma and colon cancer FFPE samples were run by DDA using a 10 min microflow gradient on a TripleTOF® 6600+ System, requiring 11 hours of MS acquisition. Each dataset was uploaded to SCIEX Cloud and searched with ProteinPilot™ Software in OneOmics™ Suite. A thorough search with biological modifications on was performed, meaning over 800 modifications were considered in the search space using feature probabilities.
Results: Searching DDA data in the SCIEX Cloud can provide improvements in search speed of 3-7x depending on the power of the desktop computer. Here, cloud searching was used to accelerate the creation of ion libraries for SWATH Acquisition data processing. From the plasma fractions, an ion library consisting of 1200 proteins with 23759 peptides at < 1% global FDR rate was created with a processing time of 1.25 hours. From the FFPE fractions, an ion library of 7269 proteins with 93949 peptides was created with a processing time of 4.7 hours. The observed median peak widths of the datasets were ~4 sec wide at half height, highlighting the quality of the chromatography that facilitated such high quality DDA datasets. Given the speed and scale of proteomics experiments today, new data processing solutions are needed to keep up with the pace of research. With fast LC-MS and cloud-based database searching, ion libraries to power SWATH acquisition studies can be generated easily in a less than a day.

P014

Simple targeted assays for metabolic pathways and signaling: a powerful tool for targeted proteomics

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Topic: Bioinformatics and Statistics

Introduction: Nowadays, protein quantification and validation are performed by using targeted mass spectrometry approaches such as selective or parallel reaction monitoring (SRM / PRM) with or without isotope dilution measurements. However, creating protein or even pathway specific assays, targeting multiple proteins remains a laborious and time-consuming task. In addition, sophisticated analyses require high-quality and high-resolution reference data to set up such targeted assays. To overcome these issues, we introduce STAMPS, a pathway-centric web service for the facilitated development of targeted proteomics assays offering a multitude of different options to design personalized and pathway centric methods usable for targeted mass spectrometry.

Method: By processing hundreds of gigabytes of high-resolution mass spectrometric data, we were able to build a database with STAMPS that currently contains 56 metabolic and 49 signaling pathways, 16,810 proteins, 116,873 unique peptide sequences and 152,000 high-resolution spectra that were manually curated. Furthermore STAMPS is accompanied by several intuitive interfaces which guide the user towards a rapid and simplified method design.

Results: Using our curated framework for signalling and metabolic pathways, we achieved a 148-fold reduction of the average development time of a pathway-specific assay in comparison to the second fastest state-of-the-art tool. Its core function is an interactive pathway interface (manually curated by domain experts) enabling the user to browse through a graph-based visualization of pathways, to (de)select proteins, to retrieve additional protein and metabolite information and to download the provided MS/MS spectra with a ‘few click’ solution. STAMPS is available as a web tool, free of charge for academic purposes and can be accessed at https://stamps.isas.de.

P016

A new algorithm for FAIMS data analysis with accurate in-depth quantitative profiling

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[1]*Bioinformatics Solutions Inc*

Topic: Bioinformatics and Statistics

Peptide identification by DDA LC-MS/MS relies on the sequential selection of precursor ions as they elute from the chromatographic column. Complex samples represent a challenge due to the presence and occurrence of chimeric tandem mass spectra from co-eluting ions. Gas-phase ion fractionation opens new perspectives to overcome sample complexity. FAIMS provides an important resource to combat this issue, however, quantitative analysis of FAIMS data remains a challenge. Here we present a new algorithm, including 4-dimensional feature detection and alignment for FAIMS data analysis. FAIMS holds the promise of delivering gas-phase fractionation by stepping through multiple compensation voltages (CVs), deconvoluting overlapping peptide signals, and removing contaminants. The new algorithm (i) groups individual CV from runs together allowing for higher precursor resolution and feature detection; (ii) uses multiple overlapping feature associations per MS/MS spectrum for in-depth identification; (iii) performs 4D feature alignment across different LC-MS/MS runs allowing peptide intensity to be calculated as summation of values in all CVs (iv) achieves TMT quantification by excluding chimeric spectra and separating MS/MS spectra from distinct CV values allowing higher accuracy quantification. The algorithm was implemented with PEAKS XPro and tested with multiple published datasets. Testing one dataset(PXD009547), this algorithm showed a marked decrease in percentage of chimeric spectra from 39% to 25% with FAIMS. This reduction in chimerism leads to significant benefits in improving proteome coverage. A 34% gain in the number of unique peptide identifications compared with traditional LC-MS/MS analyses for the same number of injections. The reduction in complexity also has significant effects in quantification. Testing a second dataset(ChousID1496), 1100 more protein groups were quantified using FAIMS, with noticeable increases in feature detection and alignment correlations, and decreased in standard deviation across injection replicates. Together, this suggests that the new PEAKS algorithm provides accurate and sensitive quantitative analysis with FAIMS data.

P017

In-Silico Construction of Homo sapiens Antimicrobial and Immunomodulatory Peptide Database DEAMP

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Topic: Bioinformatics and Statistics

Introduction:
Host defence peptides or antimicrobial peptides (AMPs) are elements of the innate immune system. They are generally gene-coded cationic peptides or small proteins being either constitutively expressed or induced to fight off potential pathogens. AMPs have role in immune modulation, apoptosis, wound healing and fertilization.
Our primary goal was to include all available data regarding human AMPs into a comprehensive database, the DEAMP. Having the data available we aimed to examine the changes in the level of human AMPs in Alzheimer's disease (AD).
Methods:
We have collected AMP data from the literature and five online available databases. Collected data were curated and organized into a database. AD protein datasets were retrieved from the ProteomeXchange repository and PubMed and the level of the AMPs listed in DEAMP were examined in the datasets.
Results:
We collected 187 human AMPs in the DEAMP database and examined their levels in 15 AD-related protein datasets. 38 AMP proteins were observed to change in a statistically significant manner in disease and 238 AMP proteins were found to be present but no statistically significant change in their level between Alzheimer's disease and controls groups could be observed.
Conclusion:
The DEAMP database was constructed and used to examine AMPs related to AD. We could detect statistically significant changes in AMP levels in the datasets generated from the analysis of brain samples, cerebrospinal fluid samples and serum sample. The number of AMPs present in the different sample types further suggest that Alzheimer's disease is rather related to a local and not necessarily to a systemic inflammation.

P018

OpenTIMS & TimsPy: Open Access to timsTOF Pro Raw Data

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Topic: Bioinformatics and Statistics

Introduction

timsTOF Pro is a novel mass spectrometer by Bruker that couples trapped ion mobility spectrometry (TIMS) together with liquid chromatography and mass spectrometry.
Collected data is stored in proprietary Tims Data Format (TDF).
The collected data is typically few gigabytes large.
OpenTIMS is the first fully Open Source solution for accessing TDF files.
TimsPy further integrates OpenTIMS with numpy, Pandas, and vaex.
Together, they bridge the gap between low level data representation and high level ease of data inspection and visualization.
All major platforms such as Linux, MacOS, and Windows, are supported.

Methods

OpenTIMS is a C++ based (with Python bindings) interface to Bruker's binary format for TIMS experiments. It can also directly covert TDF to hdf5 format in the command line.
It operates independently of Bruker's proprietary libraries.
TimsPy allows to access timsTOF Pro measurements in a tabular format.
Each row represents a particular peak and includes measurements of its retention time, ion mobility, mass to charge ratio and intensity.
The outputs can be easily processed using numpy, Pandas, and vaex modules.
Both tools are available on the Python Package Index (PyPI) and can thus be easily installed.
Source code is available on github (https://github.com/michalsta/opentims, https://github.com/MatteoLacki/timpsy).
All major platforms, including Linux, MacOS, and Windows are supported.

Conclusions
OpenTIMS&TimsPy offer free, fully open-source, quick and simplified access to timsTOF Pro data, along with basic visualization and analysis.

P019

MealTime-MS: A machine learning-guided real-time mass spectrometry analysis for protein identification and efficient dynamic exclusion

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Topic: Bioinformatics and Statistics

Introduction: While mass spectrometry-based proteomics can identify thousands of proteins in a biological sample, commonly used mass spectrometry data acquisition approaches suffer from a poor identification sensitivity of low abundance proteins. In a typical protein identification experiment, mass spectra are preferentially collected from proteins with higher abundance. The identification of these proteins is then performed after the completion of the experiment. Such an approach typically results in the redundant acquisition of mass spectra from proteins with high abundance, while very few are collected for low abundance proteins, which therefore remain unidentified.

Methods: Herein, we propose a novel supervised learning-based algorithm (MealTime-MS [1]) that identifies proteins in real-time as mass spectrometry data are acquired and prevents redundant data acquisition from already confidently identified proteins. MealTime-MS performs a Comet [2] sequence database search on mass spectra as soon as they are acquired to match them to peptides. Search results are then used by a logistic regression classifier to assess in real-time the confidence of protein identifications. Our algorithm then excludes from mass spectrum acquisition peptides that belong to confidently identified proteins by maintaining a dynamic exclusion list.

Results: Using in-silico simulations of a previously performed mass spectrometry analysis of a HEK293 cell lysate, we demonstrate that MealTime-MS successfully identifies 92.1% of the proteins normally detected in the experiment without any data exclusion, while using only 66.2% of the mass spectra. We also show that our approach outperforms a previously proposed method, is flexible and is sufficiently fast for real-time mass spectrometry analysis.

Conclusions: Finally, MealTime-MS' efficient usage of mass spectrometry resources will provide the tools for a more comprehensive characterization of the proteomes of complex samples.

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P020

Common Decoy Distribution as an alternative to dataset-specific decoy-based FDR control in shotgun proteomics

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Topic: Bioinformatics and Statistics

Introduction: In shotgun proteomics, one of the requirements for reliable peptide identification is false discovery rate (FDR) estimation. Current approaches to FDR control are dominated by empirical dataset-specific methods with assumptions frequently lacking sound theoretical basis, which may lead to variable performance and generation of inaccurate FDR estimates. In this study, a new approach to FDR estimation using the concept of a fixed empirical null score distribution based on a single-spectrum statistical confidence measure (E-value), called Common Decoy Distribution (CDD) is proposed.

Methods: CDDs for different charge states were derived from scores of over 10 million decoy human peptide-spectrum matches (PSMs). Based on Extreme Value Theory, CDDs were assumed to follow the Gumbel distribution and implemented as fixed models in parametric PeptideProphet framework. Using modified spectra, sensitivity of CDDs with respect to noise levels and presence of unexpected post-translational modifications (PTMs) was investigated and assessed by simulated FDR control. Utility of CDD-based solution was evaluated in terms of FDR estimation accuracy, retrieval of true positive PSMs, and compared with results obtained for the popular statistical validation tools, PeptideProphet and Percolator.

Results: CDDs constructed for different charge states were negligibly sensitive to changes in noise levels and presence of unexpected PTMs. Optimized PeptideProphet-based implementation of CDDs enabled calculation of satisfactorily accurate and precise FDR estimates for synthetic and natural validation datasets, with overall performance on par with that of PeptideProphet and Percolator. Precision of CDD-based FDR control improved with increase in precursor mass tolerance applied during sequence database search and, for optimal settings, was superior among the investigated tools.

Conclusions: CDD-based approach is a viable alternative to current dataset-specific decoy-based validation tools, allowing faster and simpler FDR control. Being a fixed reference point, it could facilitate a fair comparison of results of proteomic experiments conducted by different research groups.

P021

MASH Explorer, a Universal, Comprehensive, and User-friendly Software Environment for Top-down Proteomics

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Topic: Bioinformatics and Statistics

Introduction:
Top-down mass spectrometry (MS)-based proteomics enables comprehensive analysis of proteoforms with molecular specificity to achieve a proteome-wide understanding of protein functions. However, the lack of a universal software tool for top-down proteomics is becoming increasingly recognized as a major barrier, especially for newcomers. Herein, we present MASH Explorer, a universal, comprehensive, and user-friendly software environment for top-down proteomics. MASH Explorer integrates multiple spectral deconvolution and database search algorithms into a single, universal platform to process top-down proteomics data from various vendor formats(1).

Methods:
MASH Explorer is a multithreaded Windows application implemented in C# using the .NET framework within the Visual Studio integrated development environment. The software visual components are provided by Microsoft Office Runtime Support. Importing data from different MS instruments is supported using ProteoWizard, DeconEngine, and vendor-provided libraries. For the strategy of combining deconvolution results, clusters were generated using Python and the machine learning analysis was performed using R.

Results:
MASH Explorer (http://ge.crb.wisc.edu/MASH_Explorer/index.htm) is a comprehensive software environment for top-down proteomics. The core functions of MASH Explorer include spectral deconvolution, protein identification, proteoform characterization, graphical data output, data validation, and workflow automation. MASH Explorer can process data from multiple vendor formats and features workflows for discovery- and targeted-based top-down proteomics. Moreover, MASH Explorer now features a machine learning tool for spectrum deconvolution to enhance the accuracy and confidence in protein identification(2).

Conclusions:
MASH Explorer offers a universal platform for processing top-down MS data, addresses an urgent need in the rapidly growing top-down proteomics community, and is freely available worldwide. With tremendous

community support, we envision that MASH Explorer will play an integral role in advancing top-down proteomics to reach its full potential for biomedical research.

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P022

Research on The Human Proteome Reaches a Major Milestone: >90% of Predicted Human Proteins Now Credibly Detected, according to HPP

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Topic: Bioinformatics and Statistics

Summary: According to the 2020 Metrics of the HUPO Human Proteome Project (HPP), expression has now been detected at the protein level for >90% of the 19,773 predicted proteins coded in the human genome. The HPP annually reports on progress made throughout the world toward credibly identifying and characterizing the complete human protein parts list and promoting proteomics as an integral part of multi-omics studies in medicine and the life sciences. NeXtProt release 2020-01 classified 17,874 proteins as PE1, having strong protein-level evidence, up 180 from 17,694 one year earlier. These represent 90.4% of the 19,773 predicted coding genes (all PE1,2,3,4 proteins in neXtProt). Conversely, the number of neXtProt PE2,3,4 proteins, termed the "missing proteins" (MPs), was reduced by 230 from 2129 to 1899 since the neXtProt 2019-01 release. PeptideAtlas is the primary source of uniform re-analysis of raw mass spectrometry data for neXtProt, supplemented this year with extensive data from MassIVE. PeptideAtlas 2020-01 added 362 canonical proteins between 2019 and 2020 and MassIVE contributed 84 more, many of which converted PE1 entries based on non-MS evidence to the MS-based subgroup. The 19 Biology and Disease-driven B/D-HPP teams continue to pursue the identification of driver proteins that underlie disease states, the characterization of regulatory mechanisms controlling the functions of these proteins, their proteoforms, and their interactions, and the progression of transitions from correlation to co-expression to causal networks after system perturbations. And the Human Protein Atlas published Blood, Brain, and Metabolic Atlases. This paper complements "A High-Stringency Blueprint of the Human Proteome (Nat Comms, 2020) Adhikari et al."

P023

Assessment of quantitation and statistical methods for DIA mass spectrometry-based proteomics data

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Topic: Bioinformatics and Statistics

Introduction: The data-independent acquisition (DIA) approach in mass spectrometry-based proteomics has emerged as a promising alternative to data-dependent acquisition (DDA). For biomarker discovery studies, it is necessary to assess the performance of downstream analysis methods in a comparative analysis.

Methods: Three datasets were used. In the Bruderer 15 dataset [1] (n=24), 12 proteins were spiked in at different concentrations. In the Bruderer 17 datasets [2] (n=6), three species were mixed in a stable human proteome. The moonshot dataset [3] (n =120) contains a mixture of human, yeast and E. coli proteomes at different concentrations. All datasets were processed by the Spectronaut software. The R package iq [4] was employed for protein quantification, offering four different methods meanInt, topN, median polish and MaxLFQ. In our first assessment, the t-test and limma were considered for statistical significance analysis.

Results: For quantitative analysis, MaxLFQ outperforms other methods, confirming a previous result on the Bruderer 15 dataset [4]. The limma method is better than the t-test method on the Bruderer 17 datasets and equal on the moonshot dataset in terms of area under the curve.

Conclusions: The preliminary result shows that MaxLFQ protein quantification as implemented in the R package iq [4] and limma statistics provide an effective approach for biomarker discovery studies. Ongoing analysis includes assessment of the effect of sample size, missing data, preprocessing software tools, additional statistical tests and phosphoproteomics experiments.

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P024

Novel Protein-level probabilistic score and FDR estimation approach for protein identification in large-scale shotgun proteomics

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Topic: Bioinformatics and Statistics

Introduction: The target-decoy approach is the most popular method for estimating the false discovery rate (FDR) in shotgun proteomics. This method has been successfully used at the peptide level using more or less elaborated peptide-level scores, but controlling the protein-level FDR is not straightforward. A protein-level FDR can be estimated using protein-level scores, which can be computed from the scores of its identified peptides in different ways. However, empirical evidence suggest that simply using the score of the best peptide of the protein is actually more efficient, specially for large datasets (1), which is somewhat paradoxical. Here, we present a protein-level FDR approach using a novel protein-level score that uses the information of all the identified peptides and resolves this peptide-to-protein paradox.
Methods: To validate our results we have used three search engines for three tissues of the Human Proteome Map, and two protein standards.
Results: A novel probabilistic protein-level score has been developed using the information of all the identified peptides of the protein. This score accurately predicts the behavior of the decoy proteins and thus acts as a true protein probability score. Using this score to compute a protein-level FDR we provide an improved precision and sensitivity in comparison to other approaches that use the best peptide or the product of peptide probabilities. Furthermore, we also present a refinement of the picked method (2) to calculate the FDR at the protein level.
Conclusions: Our novel protein-level score and refined protein-level FDR approach can be integrated into existing protein identification workflows to improve their precision and sensitivity in large-scale shotgun proteomics analyses (3).

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P025

PointNovo: instrument-resolution-independent de novo peptide sequencing for high-resolution devices

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Topic: Bioinformatics and Statistics

De novo peptide sequencing is the key technology of finding novel peptides from mass spectra. The overall quality of sequencing results depends on the de novo peptide sequencing algorithm as well as the quality of mass spectra. Over the past decade, the resolution and accuracy of mass spectrometers have been improved by orders of magnitude and mass spectra of higher resolution have been generated. How to effectively take advantage of those high-resolution data without significantly increasing the computational complexity remains a challenge for de novo peptide sequencing tools. Here we present PointNovo, a neural network based de novo peptide sequencing model that can robustly handle any resolution levels of mass spectrometry data while keeping the computational complexity unchanged. Our extensive experiment results show PointNovo outperforms existing de novo peptide sequencing tools by capitalizing on the ultra-high resolution of the latest mass spectrometers.

P026

Integrating Prosit with Deepsearch such that Dozens of PTMs can be Potentially Searched Directly Against Deepsearch DIA Data

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Topic: Bioinformatics and Statistics

Introduction & Informatics Methods: Deepsearch, introduced at ASMS 2020, is a MS-protocol coupled to a corresponding informatics solution which attempts to maximally deconvolve MS spectra. Here, we extend Deepsearch to include a custom search engine that a) integrates Prosit's fragment intensity predictions b) parallelizes Prosit's non-GPU code c) adds MS2 m/z isotopic distributions to Prosit and merges nearly isobaric MS2 fragments d) recalibrates observed m/z data as a function of m/z, intensity and other variables e) calculates the m/z variance f) replaces the typical spectral-angle/dot-product calculations with a new comparison function based on Jakaart distances and g) modifies Prosit results to support some common PTMs.

MS Methods: 21 samples were run on Lumos with 11 iRT peptides and 76 stable isotope labelled (SIL) peptides using the globally-staggered MS DIA protocol (gs-DIA) (ASMS 2020). Briefly, instead of submitting 21 identical method files, we submit 21 method files which have slightly different DIA window starting/stopping points, which Deepearch can then deconvolve to create consensus spectra with up to 1/21th effective windows sizes.

Results: Deepsearch identified 10 of the 11 iRT peptides at an FDR much lower than 1% in all 21 samples whereas OpenSwath, which used a fractionated DDA library, only found 7 of the 11 iRT peptides in all 21 files and 2 of the 11 in only about half of the 21 samples. Of the 76 SIL peptides only ~50 SIL peptides were detectable by a *targeted* Skyline approach ("ground-truth"). Deepsearch identified those 50 but at a PTM-specific FDR of ~10%, whereas a DIA-Umpire pipeline (using Comet and X!Tandem) detected 50 SIL peptides but at a PTM-specific FDR of >> 500%. By September-end, we hope to have searched dozens of biologically interesting PTMs simultaneously but with PTM-specific FDRs that are practical to a scientist (eg ~5% instead of >> 500%).

P027

Characterization of peptide-protein relationships in protein ambiguity groups via bipartite graphs for an improved protein quantification

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Topic: Bioinformatics and Statistics

Introduction:
In bottom-up mass spectrometry proteins are digested to peptides before LC-MS measurement, usually using trypsin. Therefore, protein inference must be conducted and peptide quantities have to be summarized to protein quantities. This is a complex task due to shared peptides and leads to the formation of protein ambiguity groups. The relationship between proteins and peptides can be represented by bipartite graphs, where two types of nodes represent proteins respectively peptides and there is an edge if the peptide sequence is part of the protein sequence. This representation is useful to investigate the full potential for protein inference and quantification.
Methods:
Two different datasets (mouse respectively yeast background with spiked-in proteins) and the corresponding protein databases were used to create bipartite protein-peptide graphs. The graphs were generated on the level of all possible peptides (in silico digestion of the databases) as well as on the quantified peptides only. The graphs were split into connected components, i.e. graphs containing groups of proteins that are connected by chains of shared peptides. Isomorphism classes, i.e. groups of isomorphic graphs, which have the same basic structure, were formed.
Results:
Connected components from the same isomorph class have the same or similar properties regarding the inference or quantification of the corresponding proteins. The resulting connected components differ between databases from complex (mouse) and less complex (yeast) organisms by number, size and types of occurring isomorphism classes. The composition of isomorphism classes changes when only quantified peptides are considered, which has an impact on the inference and quantification of proteins.
Conclusions:
Analysis of the bipartite protein-peptide graphs are useful to aid the protein inference and quantification process. In particular the consideration of the graphs in their entirety over the whole dataset or database will give indications for an improved quantification, especially for proteins without unique peptides.

P028

A toolkit for the quantitative behavior of protein complexes

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Topic: Bioinformatics and Statistics

Introduction:
Protein complexes regulate crucial cellular processes in the cell. These molecular machines allow a high functional diversity due to their ability to change their composition and structure. In addition, they are among the most tightly controlled entities by selective degradation of their individual proteins and thus create specific footprints in proteomics data. Improved characterization of their functional states requires methods for the characterization of complex composition, behavior and abundance across multiple cell states.
Methods:
We developed a user-friendly software toolkit for statistical and in-depth investigation of the behavior of protein complexes in quantitative proteomics data. Appropriate and extensive randomizations were used to provide accurate measures for significant co-regulation of protein complex members in human cells, based on protein measurements across >200 cell types provided in the ProteomicsDB repository. Quantitative changes of protein complex abundance are estimated by factor analysis.
Results:
The tools CoExpresso and ComplexBrowser allow interactive supervised analysis of protein complex behavior in any quantitative proteomics data, with comparison to general behavior in human cells. The power of this analysis is shown for experimental data from differentiation of satellite cells into muscle cells.
Conclusions:
Proteomics data contains a usually untapped treasure of information about protein complex behavior which now can be accessed by the tools CoExpresso and ComplexBrowser.

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P029

BioInfra.Prot: Bioinformatics Services for Proteomics

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Topic: Bioinformatics and Statistics

Introduction: The service center BioInfra.Prot, which is part of the publicly funded German Network for Bioinformatics Infrastructure (de.NBI, https://www.denbi.de/) and the European bioinformatics infrastructure network ELIXIR (https://elixir-europe.org/), provides a comprehensive portfolio of bioinformatics services for proteomics.

Methods: Due to the strong need for bioinformatics infrastructure in proteomics research, we focus on providing free high quality services to the proteomics user community. Our service portfolio includes a number of bioinformatics services from various categories such as software tools, pre-defined workflows for typical proteomics tasks, teaching as well as data analysis and consultancy.

Results: Here, we present an overview of our services. Altogether, we offer a comprehensive proteomics workflow. This workflow includes data standardization, data conversion and data publication services as well as computational and statistical consultancy and data analysis, software tools, the biomarker database BIONDA and training courses. Since project start in 2015, we contributed as co-authors to more than 50 scientific articles and organized more than 20 training events. We recorded more than 13,000 service tickets and more than 10,000 software downloads.

Conclusion: The relatively high usage of our free and high-quality services as well as the high user satisfaction confirms the strong need for bioinformatics infrastructure in proteomics research.

P030

Database for fast weight-based peptide search

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Topic: Bioinformatics and Statistics

Introduction: The current UniProt (1) release contains 181 million searchable proteins with a lot of information besides the protein sequence alone like gene name, organism, taxonomy and function. Despite this impressive number, all bottom-up proteomics methods do not only rely on protein information but actually on peptides. The peptide search of UniProt though is rather slow: A single peptide needs several seconds. Using Unipept (2), the search is much faster, but the information lacks some important information for e.g. SRM/MRM, like the peptide weights.
Methods: Using modern server hardware, the newest PostgreSQL release and a highly concurrent implementation for digestion, we are able to digest all proteins contained in the UniProt in about 12 days. Hereby we allow up to two missed cleavages and a peptide length of 6 to 50 amino acids. We also take advantage of the fact, that Leucine and Isoleucine are undistinguishable by MS methods and replace them with their common one letter code 'J'. The database schema was optimized by partitioning the peptide table into 100 balanced chunks to speed up database maintenance.
Results: A database containing 5.9 billion tryptic peptides with information about their weight, length, number of missed cleavages (up to two) and protein affiliation including taxonomy information was created. To simplify requesting the database, a graphical web interface was designed.
A search for a peptide sequence or for a weight to retrieve all sequences is performed in milliseconds. Each search can be refined with modification, mass tolerances and taxonomy. With the provided web API the database could be integrated in existing workflows.
Conclusion: The created database containing all tryptic peptides of UniProt may help by designing SRM/MRM, PRM or further proteomics studies, which require additional peptide and peptide weight information, and is ready to be used.

P031

Optimization of timsTOF Pro data processing for large sample cohorts by concurrent processing during acquisition on PEAKS Online Platform

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Topic: Bioinformatics and Statistics

Introduction
With the recent advance in the mass spectrometry instrumentation technology, the latest mass spectrometers can acquire data with much higher speed and resolution. This puts a lot of pressure on the software to analyze the data in parallel to instrument acquisition. Especially with the publication of the timsTOF Pro instrument by Bruker, one can acquire more than a hundred MS/MS scans per second, with ion mobility as an additional dimension. To analyze this new type of data, we propose a new software structure based on the well-known PEAKS Online platform. With the new software structure, the data acquisition and analysis will run in parallel. The analysis result can be acquired as soon as the acquisition is finished.

Methods
PEAKS Online is a distributed computing platform for MS data analysis. A PEAKS Online daemon program is developed and deployed on the instrument computer. On the PEAKS Online server, we setup a project. In this project, we specify for each sample which instrument their data should come from. Then these message are sent to the daemon program. A project-sample structure directory are generated on the instrument computer. The daemon program scans this directory to decide whether the data has been acquired. Once the data has been acquired, it will be sent to the PEAKS Online server. The analysis procedure starts immediately when the data is received by the server.

Results
The final database search results were acquired 4 hours after the last LC-MS run was finished. The same database search analysis was performed again on the same server after all the data were acquired. This analysis took around 200 hours. The concurrent processing reduced the waiting time for the final results from 200 hours to 4 hours.

Conclusions
Concurrent processing during acquisition to significantly reduce processing time for large sample cohorts.

P032

Prediction of peptide spectral libraries by deep learning facilitate data-independent acquisition proteomics

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Topic: Bioinformatics and Statistics

Introduction: Data-independent acquisition (DIA) is an emerging technology for quantitative proteomic analysis of large cohorts of samples. However, sample-specific spectral libraries built by data-dependent acquisition (DDA) experiments are required prior to DIA analysis, which limits the identification/quantification by DIA to the peptides identified by DDA. It is of great value to generate in silico spectral libraries with quality comparable to that of experimental libraries for DIA analysis.

Methods: We constructed deep neural networks for accurate peptide MS/MS spectra and retention time prediction, which is used to generate in silico spectral libraries for DIA data analysis. The models take a peptide sequence as an input, and outputs relative intensities of b/y product ions at each possible fragmentation site, as well as normalized retention time (iRT) of the peptide. Deep learning was also used to predict detectability by mass spectrometry of peptides, and in silico libraries can be built directly from protein sequence databases.

Results: We trained and validated the model with three LC-MS/MS data sets of two organisms acquired in two laboratories. The change of lab gave higher impact on the accuracy of prediction than the change of organism, and good cross-sample prediction is feasible when keeping the instrument same. We demonstrate that the quality of in silico libraries predicted by instrument-specific models is comparable to that of experimental libraries, and outperforms libraries generated by other deep learning-based tools using global models. With peptide detectability prediction, in silico libraries can be built directly from protein sequence databases.

Conclusions: Our strategy can break through the limitation of DDA on peptide/protein detection, and enhance DIA analysis compared to the state-of-the-art protocol using a DDA library.

P033

Evaluation of Six Software Tools for Data-independent Acquisition Mass Spectrometry-based Proteomics

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Topic: Bioinformatics and Statistics

Introduction: Several software tools have been developed to analyze the rapidly growing data-independent acquisition (DIA) mass spectrometry (MS)-based proteomic data sets. The performance of some tools has been evaluated several years ago, but the state-of-the-art performance of these tools and their consistency remain unclear.

Methods: Our evaluation was based on several benchmark data sets. We firstly analyzed with the publicly available datasets which are composed of multi-species peptide digests using fixed ratios as indicators for ground truth, generated by TripleTOF6600, Orbitrap QE HFX, and TimsTOFPro instruments. We also generated HeLa cell digests acquired with different gradient length using TimsTOF Pro. A DIA data set of testis cancer and nearby tissue samples measuring up to 10,000 proteins groups in a Q Exactive HFX mass spectrometer was also analyzed. The datasets were analyzed with DIA tools including OpenSWATH, EncyclopeDIA, Skyline, DIANN, Spectronaut, and PEAKS.

Results: We found about 50% proteins of relatively high abundance were identified by all six DIA software tools, while the relatively low abundance ones were detected by a subset of the DIA tools. For TripleTOF data, Spectronaut identified highest number of proteins, while DIA-NN identified highest number of proteins from Orbitrap data. In pairwise comparisons of these software tools in terms of protein quantification, our data showed that the retention time of peak group apex was relatively consistent (r=0.98), while the abundance values were less consistent among different tools (r=0.82), suggesting that these tools may use different strategies to report quantitative values. Differentially expressed proteins commonly identified by six tools were those with highest fold-changes. More details will be discussed.

Conclusion: Different DIA tools shared high degree of consistency in protein identification but the quantification showed discrepancy. More in-depth analyses are needed.

P034

Proteomic glimpse at the M2-polarized microglia cells and their role in tumor progression

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Topic: Brain and CNS Diseases

Introduction: Microglia safeguard the CNS against pathogens by inducing an inflammatory response. In response to anti-inflammatory cytokines, however, these cells possess the ability to switch from an inflammatory M1 to an immunosuppressive M2 phenotype. Cancer cells exploit this property to evade the immune system and elicit an anti-inflammatory microenvironment that facilitates tumor attachment and growth. In this study, we used proteomics and mass spectrometry technologies to shed light on the biological processes that are activated in microglia cells in response to anti-inflammatory cytokines released from cancer cells.

Methods: Serum-depleted and non-depleted human microglia cells (HMC3) were treated with a cocktail of anti-inflammatory cytokines including IL-4, IL-13, IL-10, TGF-β and the chemokine MCP-1 for 24 hours in culture. The nuclear and cytoplasmic cell fractions were analyzed by LC-MS/MS using a QE-Orbitrap mass spectrometer. The experiment resulted in the identification of a total of ~10,000 proteins. Using functional annotation clustering tools, statistically significant proteins that displayed a >2-fold change in abundance between cytokine-treated and non-treated cells were mapped to their biological networks and pathways.

Results: Cytokine stimulation elicited metabolic changes in the microglia cells which caused an up-regulation of mitochondrial proteins that carry out oxidative phosphorylation processes. The increase in mitochondrial activity was positively correlated with an over-expression of collagen and other ECM remodeling proteins which are crucial for creating a tumor-supportive niche. PPI networks revealed that these changes were tightly controlled by the regulatory proteins of the MAPK cascade. The suppression of cell-cycle progression and transcription proteins, possibly mediated by IL-10 and TGF-β, limited the inflammatory activation of microglia towards the M1 phenotype.

Conclusions: The comprehensive proteomic profiling of the HMC3 cells with the perspective of their cancer promoting phenotype provided novel insights into the molecular mechanisms that drive cancer development in the brain and may prove beneficial for therapeutic studies.

P035

Multiomics profiling of Alzheimer's Disease for the identification of serum autoantibody biomarkers

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Topic: Brain and CNS Diseases

Introduction

Alzheimer's disease (AD) is the most common cause of dementia worldwide with a high socioeconomic impact. Because the definitive diagnosis of AD requires post-mortem verification, new approaches to study AD are necessary to get further insights into the disease. Here, we aimed to identify AD-specific autoantibodies and their target proteins using PrEST protein arrays and mass spectrometry-based methods.

Methods

The untargeted screening of AD autoantibodies was performed using high-density (42,000) and low-density (384) PrESTs planar arrays, by which more than 42,000 PrEST antigens were analysed, and an immunoprecipitation protocol coupled to mass spectrometry analysis for serum autoantibody profiling against native brain proteins. Validation of the results was performed using antigen suspension bead arrays, ELISA, immunohistochemistry, and luminescence Halotag-based beads immunoassay.

Result and discussion

A total of 370 unique PrESTs corresponding to 338 targets from the screening phase were used for validation by antigen suspension beads arrays. In addition, among the identified proteins target of autoantibodies 10 were selected for validation, showing a higher seroreactivity in AD than in controls. Among identified PrESTs target of autoantibodies, a candidate PrEST was found with a statistically significant higher seroreactivity to AD patients in comparison to controls. In addition, this target was further validated as full-length recombinant protein by ELISA. The significant dysregulation in AD prefrontal cortex tissue of this candidate biomarker was observed by WB and immunohistochemistry using tissue microarrays.

Conclusion

Our results suggest that the combination of microarrays and mass spectrometry-based methods is useful for the identification of autoantigens specific of AD and protein alterations related to the disease. Although complementary immunological approaches are needed for further validation of the identified proteins target of autoantibodies to verify their diagnostic ability, the identified panel of AD autoantibodies provides new insights into the blood-based diagnosis of the disease.

P036

Cause and effects of H2S-mediated redox unbalance in ALS proteome

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Topic: Brain and CNS Diseases

Introduction: Hydrogen sulfide (H_2S), a toxic gaseous molecule, acts as cell-signalling factor in the central nervous system to maintain a balanced amount of antioxidants and combat oxidative species. However, H_2S may have a double biological action switching from neuromodulator to neurotoxic when present at harmful levels. In addition, H_2S mediates the formation of cysteine persulfides (R-S-SH) which affect the activity of target proteins in determining susceptibility to oxidative damage and neurodegeneration.
Toxicity mediated by aberrant H_2S levels has already been shown in Amyotrophic Lateral Sclerosis (ALS). The mechanisms underlying this anomalous production need to be clarified. This study aims to investigate both the origin of such altered H_2S levels in ALS and its effects in ALS redox proteome.
Methods. Dedicated mass spectrometry-based proteomics analysis have been developed to quantify H_2S-producing enzymes and to enrich and identify cysteine persulfides. Proteomics analysis was performed on total extract and mitochondrial enriched fractions derived from spinal cord of ALS SOD1-G93A and SODwt mice. Both approaches, LC-MS DDA on Orbitrap Elite mass spectrometer (Thermo) and high definition DIA approach on Synapt G2Si Q-TOF mass spectrometer (Waters) have been carried out. DDA and DIA MS data have been processed independently using Peaks studio and PLGS softwares, respectively. Quantitative analysis was performed using Skyline. Bioinformatics analysis was performed on Ingenuity Pathway analysis software (IPA, Qiagen).
Results. An altered expression of H_2S producing enzymes was shown in ALS tissues, depending on its location. In addition, site-specific identification and quantification of cysteine persulfides highlighted proteins differently expressed in SOD1-G93A compared to SODwt.
Conclusions: H_2S itself seems to control a feedback regulation of enzymatic expressions in ALS. Moreover, the adaptation of ALS redox proteome with persulfides especially involved in oxidative stress pathways may contribute to amplify ALS pathogenesis.

P037

CESI-MS of neuronal cultures differentiated from ALS patient-derived iPSCs, reveals differentially regulated PTMs and biological pathways.

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Topic: Brain and CNS Diseases

Introduction: ALS is a devastating neural degenerative disease with no cure or current treatment standard and is often fatal within 5 years of diagnosis. Analysis of human motor-neurons differentiated from patient-derived iPSCs may provide new insight into the biology of human neurons and protein signaling networks.
Method: Motor neuron cell cultures of patient differentiated iPSC lines were lysed in Urea and digested. CESI-MS was conducted on a CESI 8000 Plus system couple with a TripleTOF® 6600 mass spectrometer (SCIEX). Digested samples were prepared in 100 mM ammonium acetate pH 4 (leading electrolyte). Samples were injected onto 91 cm Neutral capillary, 30 µm ID (SCIEX) loading 45-70 ng of protein using 10% HOAc as BGE.
Results: CESI-MS experiments of 10 ALS cell lines and 9 control cell lines increased unique phosphopeptide identification by 58% compared to LC-MS analyses of whole proteome extracts. On average, over 200 phosphopeptides and more than 200 acetylated peptides were identified in a single CESI-MS run. Interestingly, many of the modified peptides were found to be dysregulated between disease and control samples. Protein Interaction Network Extractor (PINE), a user-friendly tool developed in-house, illustrates dysregulated protein networks identified from peptide fold changes between ALS and control samples. Microtubule-associated protein 1B (MAP1B), known for its role in neuron development, had the most phosphorylation sites identified on a single protein. The kinase prediction tool, NetworKIN was used to determine if multiple sites were predicted to be modified by the same kinase in order to associate any potentially co-regulated phosphorylated residues.
Conclusions: Changes in protein regulation by phosphorylation and not overall protein expression abundances was detected in young, developing day 18 neuronal cultures and thus may provide a list of potential diagnostic markers for ALS disease predictions. Further, CESI proves to uniquely exploit this defining sub-phosphoproteome without requiring enrichment.

P038

Distribution of Epimerized Amyloid beta Isoforms in the Sporadic Alzheimer's Disease

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Topic: Brain and CNS Diseases

Introduction
Extracellular amyloid plaques and intracellular neurofibrillary tangles are the pathological hallmarks of Alzheimer's Disease (AD). It takes on average 19 years for amyloid β (Aβ) peptides to deposit as insoluble plaques from onset to clinical dementia symptoms in AD. Long-lived nature of amyloidosis predisposes the polypeptide chains to undergo multiple spontaneous and non-enzymatic post-translational modifications (PTMs), which can render them resistant to normal cellular proteolysis mechanisms. Several biochemical/analytical approaches have estimated very high degree of epimerization of Asp and Ser residues in Aβ purified from the insoluble plaques. However, comprehensive characterization of these epimers as well as their quantitative estimation is yet to be done.

Methods
Freshly frozen post-mortem temporal cortex samples from non-demented brains (n=10) and AD brains (n=11) were homogenized and fractionated into distinct soluble, vesicular, membranous and insoluble/aggregated biological pools of Aβ. Brain homogenates were enzymatically digested and separated on liquid chromatography coupled to ESI-drift tube ion mobility (LC-DTIM) mass spectrometer. Stable isotope labeled peptides (SIS) were spiked in these brain homogenates to quantify the amount of these epimers.

Results
We have quantified the most relevant epimerized Aβ isoforms from AD brains. Epimer separation was achieved by LC and their collisional cross section values. Significant isomerization was observed in AD compared to controls brains. Moreover, we found the extent of isomerization in the soluble and peripheral membrane was lower compared to higher levels (~ 85 %) in the insoluble/debris in AD brain.

Conclusions
Isomerized Aβ peptides are intricately associated with the accumulation amyloid in the brain, allowing differentiation between old plaques vs diffuse plaques. These findings have implications in Aβ neurotoxicity, oligomerization, structures of amyloid fibrils present in the AD brain. Specifically targeting these isomers for enhanced clearance from the CNS would be crucial in developing any therapeutics targeting Aβ.

P039

Creation of truncation specific Dicer1 antibodies by a cross-discipline pipeline

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Topic: Cancer

Introduction:
While lung cancer is the leading cause of cancer deaths worldwide, current mouse models of lung cancer rarely become metastatic as is typical of human lung cancers (1). To address this gap, JAX is creating models with various human-found mutations in Dicer1, which combined with other oncogenic alleles increases metastatic tumor formation. However, Dicer1 commercially available antibodies showed poor analytical response. Therefore, a pipeline was created to produce, study, and create monoclonal antibodies for these mutant forms of Dicer1.

Methods:
The C-terminal of Dicer1 was cloned into pMal-C5X. The Dicer1 fragment was purified by amylose beads followed by SEC. The protein's sequence was verified by both bottom-up LC-MS/MS analysis for peptide sequence coverage as well as intact protein molecular weight determination. Additionally, LC-MS/MS analysis was interrogated for E. coli peptides to determine the level of purity. Monoclonal antibodies to Dicer1 C-terminal were generated. The top 10 candidates as observed by ELISA were tested for use in histological applications.

Results:
MalE-Dicer1- showed the anticipated 68.3kDa molecular weight. After tryptic digestion and LC-MS/MS analysis, protein sequence coverage was 88% with only three missing peptides. Confirmation of the intact sequence and only minor E. coli peptide contamination resulted in several high-quality antibody candidates being generated. Candidates were tested in several approaches and two were shown to recognize only the desired mutant form. This monoclonal antibody was used to confirm the presence of truncated Dicer1 within metastatic lesions in a new mouse model of human lung cancer.

Conclusions:
By the creation of a novel antibody for the study of human lung cancer in mouse models, this pipeline demonstrates the utility of close working relationships between several protein disciplines to study unique protein conundrums.
References: R. L. Siegel, K. D. Miller, A. Jemal, Cancer Statistics, 2017. CA Cancer J Clin67, 7-30 (2017).

P040

Novel Recombinant Protein SWATH/DIA Library Allows Detection of Low Abundance Protein Biomarkers from Colorectal Cancer Patient Plasma

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Topic: Cancer

Introduction: Human plasma is the most informative, accessible biofluid for assessing the status of human health. However, detection, identification and quantification of low abundance cancer-related proteins (e.g., CEA, cytokines) remains one of the principal challenges in proteomic biomarker discovery - due to high abundance plasma proteins obscuring biomarkers. Here, we employ a novel recombinant protein SWATH/DIA library approach to identify low abundance cancer-related plasma proteins.

Methods: We have generated a DDA spectral peptide library derived from tryptic cleavage of 36 human recombinant proteins. This set of proteins are previously suggested as plasma-based cancer biomarkers from literature (CEACAM5, EGF/EGFR, IL6, IL8, CPQ, MMP2/3/9, KLK3, MUC1, S100A8/9, TIMP1, TIMP2, TNF-α, TP53) or implicated from our studies (ADAMDEC1, C1QC, ITGB6, PLAUR). The constructed recombinant protein spectral library (rPSL) was coupled to SWATH-MS analysis to determine if it was possible to observe tryptic peptide spectra of these 36 cancer-related proteins in non-depleted colorectal cancer (CRC) plasma samples. To mitigate the FDR concerns due to the use of a small library, the rPSL was merged with a larger plasma protein library containing 742 proteins and SWATH-MS protein identification was performed.

Results: Of the 36 biomarkers, we reliably identified 32 proteins from non-depleted CRC plasma. Of those, 8 proteins (e.g., BTC, CXCL10, IL1B, IL6, IL8, TGFα, TNFα, TP53) had not, to our knowledge, been previously detected with any MS technologies in human plasma. Our novel rPSL approach allowed identifying very low abundant cancer-related plasma proteins that are present in the pg/ml concentration range (e.g., CEACAM5, IL8, PTEN, TGFα, TP53). The SWATH analysis on a merged library permitted to detect 3 additional proteins that were not originally identified from the rPSL-only approach.

Conclusion: We propose the use of recombinant protein SWATH libraries for the discovery of diagnostic, prognostic and theragnostic protein biomarkers for cancer/s and other diseases.

P041

Identifying interaction partners and substrates of the oncogenic deubiquitinating protein USP7 in colorectal cancer

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Topic: Cancer

Introduction: The ubiquitin-specific protease USP7/HAUSP is a deubiquitinating peptidase that cleaves ubiquitin chains from its substrates and therefore rescues them from proteasomal degradation. USP7 abundance and stability plays a major role in regulating various cellular processes and USP7 regulates multiple oncogenic pathways by maintaining the stability of key oncogenic proteins. The aim of this study is to comprehensively identify USP7 targets in colorectal cancer through mapping of the USP7-associatied protein network.

Methods: This study utilised multiple approaches to characterise the USP7 protein functional network in colorectal cancer cells. Inducible siRNA was used to knock-down USP7 in LS188 colorectal cancer cells and quantitative label-free liquid chromatography-dual mass spectrometry (LC-MS/MS) was performed using this model with EasySpray PepMap coupled to Thermo-Finnigan Orbitrap mass-spectrometry.

Results and Discussion: USP7 knockdown significantly decreased cell viability and growth. It also decreased the abundance of known USP7 interaction partners, including DNA methyltransferase I (Dnmt1) and Small ubiquitin-related modifier 1 (SUMO 1), while increasing the abundance of p53. Our LC-MS/MS identified and quantified over 4800 proteins in colorectal cancer cells, and this enabled the identification of many proteins of which the abundance was significantly altered in USP7 knockdown cells. Conclusions: Our findings open new avenues to explore USP7 as a potential target for the treatment of colorectal cancer.

P042

Identification of protein biomarkers for prediction of response to platinum-based treatment regimens in non-small cell lung cancer

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Topic: Cancer

Introduction
The majority of stage IIA-IIIA non-small cell lung cancer (NSCLC) patients eligible for surgery are treated with adjuvant platinum-based chemotherapy (ACT) in a one-size-fits-all approach. However, a significant number of patients do not benefit due to acquired or intrinsic drug resistance. No predictive patient selection biomarker is currently available for ACT. Therefore, the aim of this study was to identify protein markers that can aid in pinpointing NSCLC patients likely to derive clinical benefit from ACT.

Methods
Using global, label-free GeLC-MS/MS-based proteomics, we have profiled archived primary tumor resection material (FFPE) of 45 NSCLC patients who received ACT, and then correlated protein expression profiles to clinical outcome in order to develop a predictor of recurrence-free survival (RFS).
Using the same methodology, an independent validation cohort consisting of 32 patients that either received ACT or were left untreated following resection, was profiled. Most promising ACT response prediction candidates were identified by data integration.

Results
Proteomic profiling of 45 NSCLC patient samples identified almost 5000 proteins. Unsupervised cluster analysis revealed a poor response/survival sub-cluster that displayed a strong epithelial-mesenchymal transition (EMT) signature and high stromal score. Cisplatin sensitivity and resistance profiles beyond this stromal subpopulation were defined by applying beta-binomial statistics. We identified and validated proteins involved in pathways relevant to the mechanism of action of cisplatin, such as RNA processing, DNA damage repair and drug metabolism.

Conclusions
In the current study, protein profiles reflecting sensitivity and resistance to cisplatin were identified in 2 independent, multi-center cohorts of NSCLC patients in the ACT setting. Predictive and prognostic potential was determined by analysis of untreated resection samples. In addition, potential novel drug targets were identified. Currently, candidate predictive platinum-drug response proteins are being validated using immunohistochemistry (IHC) as an independent, clinically applicable protein assay.

P043

Transgelin contributes to a poor response of metastatic renal cell carcinoma to sunitinib treatment

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Topic: Cancer

Introduction. Renal cell carcinoma (RCC) represents about 2-3% of all cancers with over 400,000 new cases per year with an increasing incidence worldwide. Sunitinib, a vascular endothelial growth factor (VEGF) tyrosin kinase receptor inhibitor, has been used for first-line treatment of metastatic RCC (mRCC) with good or intermediate prognosis. About one third of mRCC patients, however, do not respond well to sunitinib. The aim of presented pilot study was to find proteins associated with poor sunitinib response.

Methods. 8 vs. 8 RCC tumors from patients responding vs. non-responding to sunitinib 3 months after the treatment, and their adjacent normal tissues, were analyzed using LC-MS proteomics in data independent acquisition (DIA) mode on Impact II LC-MS system (Bruker). The most promising protein was functionally analyzed via CRISPR/Cas9 technology in 786-0 RCC cell line and using proliferation tests.

Results. Proteomics analysis quantified 2012 protein groups (FDR<0.01) and revealed 42 proteins deregulated in primary tumors non-responding vs. responding to sunitinib. Gene set enrichment analysis showed enriched elastic fibres, vesicular transport and transport of small molecules pathways. Based on the above, transgelin, a well-known structural protein involved in actin remodeling, was selected for functional analysis. TAGLN gene was successfully disrupted by CRISPR/Cas9. The cells with reduced level of transgelin then exhibited significantly slower proliferation and worse survival.

Conclusion. DIA proteomics analysis revealed a pattern of deregulated proteins possibly directly or indirectly contributing to sunitinib resistance. The data shows that transgelin supports survival of RCC cells and could contribute to their intrinsic sunitinib resistance.

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P044

In search of Retinoblastoma protein family: protein enrichment and detection by targeted MS analysis in breast cancer derived cell lines

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Topic: Cancer

Introduction: The RB (Retinoblastoma-associated protein) family (RBF) is composed of three low abundant proteins: RB1, p107 and p130. Phosphorylation patterns of those proteins regulate the cell cycle, especially allowing the passage from G0 to G1 phase. It was recently shown, that phosphorylation of RB1 might play a crucial role in patient treatment with palbociclib – a CDK inhibitor used together with endocrine therapy in patients with ER+/HER2- breast cancer. The goal of this project is to develop an MS-based proteomic approach for analysis of the RB family proteins and their phosphorylation pattern in cell cultures, xenographs and ultimately in biopsies from living patients.

Methods: A range of enrichment strategies was applied: 1. a variation of detergent dependent fractionation performed on centrifugal filters, for nuclei purification; 2. a GELFrEE Fractionation; 3. MS compatible immunoprecipitation. Protein samples were digested with FASP or SP3 protocol. For phosphorylation investigation PPQ (Phosphatase-based Phosphopeptide Quantitation) and MOAC (Metal Oxide Affinity Chromatography) approaches were used. Samples were analysed by PRM or MRM (parallel or multiple reaction monitoring) MS methods against carefully picked tryptic peptides and their stable isotope standards.

Results: The GELFrEE approach was not successful, with all of the RBF proteins being below the detection level. Organelle fractionation resulted in partial sequence coverage, but the IP gave the best results. It allowed to detect and quantify not only RB1 (about 4 ng of protein starting from 1·10^6 cells) but also pS(249) (~40% of total protein), a phosphorylation site involved with palbociclib activity.

Conclusions: RBF targeted MS proteomics allowed detection and quantitation of RB1 and its phosphorylation. Currently, the method is being expanded to cover also p107 and p130 and prepared for tests on other biological samples.

P045

Identification of the prostate cancer exosome surface proteome and its application to detect prostate cancer in urine

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Topic: Cancer

Prostate cancer (PCa) cells secrete extracellular vesicles (EVs) in biofluids including blood and urine. Determination of PCa-associated EVs in biofluids might provide a minimally-invasive diagnosis method for PCa. To specifically capture PCa-related EVs, an understanding of the EV surface protein constituents is essential.

Ultracentrifuge-isolated EVs from PCa cell lines (PC3 and DU145) were treated with trypsin, after which the surface peptides were separately quantified from the luminal fraction using label-free LC-MS/MS and bioinformatics analyses. To explore clinical relevance, selected candidates were measured by ELISA in 32 PCa and control urine samples.

We identified >2000 proteins in total EV, and 411-659 proteins in the surface fraction, for PC3 and DU145, respectively. 90% of the surface proteins were present in total EV, and ~30% of total proteome consisted of proteins located on EV surface. Gene ontology analysis on the ~80 surface unique proteins revealed the presence of DNA/RNA-binding and RNA splicing protein complexes on the EV membrane($p<0.001$).

Topology analysis mapped 30% of the transmembrane surface proteins to have their intracellular domains outside, revealing bi-directional positioning of proteins on the EV membrane. To determine whether surface proteins are detected in PCa patients, we evaluated their expression in our urinary EV proteome dataset. ~20 surface proteins were significantly enriched in PCa urinary EVs, suggesting a clinical value for the EV surface proteins identified in vitro. To further study their clinical applicability, we selected 2 candidates ($p<0.05$; fold change>2) for ELISA measurement in 4 ml unprocessed urine. The 2 candidates showed an increased expression in an independent cohort of 32 PCa urine samples.

The identified preliminary PCa EV surface proteome may have implications in EVs' biological function and provides novel insights that might ultimately translate to a minimally-invasive detection method for PCa. Future studies will focus on PCa specificity of the identified surface proteome. (IMMPROVE-KWF(#EMCR2015-8022))

P046

Mapping prostate cancer using a multi-omic approach, combining data independent and targeted strategies

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Topic: Cancer

Prostate cancer is the second most commonly occurring cancer worldwide in men with 1.3 million cases reported in 2018. The PSA (prostate specific antigen) assay is routinely employed for detecting prostate cancer at an early stage but is controversial, since an elevated level of the protein does not necessarily correlate with developing the disease and could potentially be indicative of a benign, enlarged prostate and therefore additional markers need to be identified. Here, we demonstrate a multi-omic approach for a large cohort, which have been collected and analysed to reveal markers which could provide insight into the underlying biology and stratification of prostate cancer patients.

Plasma samples (n=509) collected from a cohort consisting of seven groups (control, benign prostate hyperplasia, active surveillance (detection of cancer but no treatment), radical prostatectomy treatment, brachytherapy treatment, hormonal therapy treatment, combination of hormonal therapy and radiotherapy treatment). Plasma were collected before and after treatment for all patients. Lipidomic samples were analysed using a rapid, targeted LC-MS approach, separating lipids over an 8 minute gradient. Proteomic samples utilised a discovery DIA workflow, incorporating ion mobility (IM) with peptides chromatographically separated using a high throughput approach.

Multivariate statistical analysis of the lipid data revealed separation between the various conditions, using unsupervised PCA models. Key lipids were identified, including those which are synonymous with markers of inflammation and lipid transportation processes. Specific lipid classes also provided discrimination between patients exhibiting a large prostate and those who develop cancer over various stages.

High throughput proteomics resulted in approximately 300 proteins being identified with differential regulation exhibited for a significant number of protein groups, complimenting the targeted lipidomic data. Biological significance was established by performing pathway analysis. A number of highly correlating pathways and networks were identified, including those related to cell cycle regulation and development biology.

P047

Mitochondrial Functional Dependence of Metastatic Melanoma Revealed by Proteomic Screening of a Large Cohort of Samples

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Topic: Cancer

Introduction: Malignant melanoma is the most aggressive type of skin cancer and develops from the melanocytes. Of all diagnosed cancers in Europe, 3.4% are melanomas, and up to 20% of these patients lethally relapse following treatment. Recently, the role of mitochondria in metabolic homeostasis in cancer cells and particularly in melanoma has been highlighted.

Methods: The mitochondrial proteome dynamics was analyzed by quantitative proteomics in a cohort of 151 melanoma tumors coming from 74 postmortems and 77 biopsies. Functional enrichment analyses were performed in Perseus and DAVID bioinformatics platforms. The main findings were validated at the transcriptomic level in 443 melanoma tumors from the TCGA repositories.

Results: Melanoma tumors and to a less extent in the microenvironment, upregulated the mitochondrial oxidative phosphorylation, TCA cycle and the mitochondrial translation compared to non-tumor biopsies, while downregulated the amino acid and lipid metabolism. Higher proliferative tumors upregulated the mitochondrial translation and downregulated the amino acid and lipid catabolism. The mitochondrial proteome showed variation relative to age and overall survival of the patients. Tumors from patients with short survival and older showed a metabolic shift toward the upregulation of the TCA cycle and oxidative phosphorylation while repressed pathways linked to the immune response. Metastases that developed during or after therapy upregulated the mitochondrial translational machinery and the oxidative phosphorylation. In addition, in both proteomics and transcriptomic, the levels of mitochondrial ribosomal proteins were upregulated in tumors where the disease progressed after therapy. In melanoma tumors carrying the mutation BRAF V600E we found upregulation of the oxidative phosphorylation in both the proteomic and transcriptomic data.

Conclusions: Our results revealed mitochondria as an important player in the development and progression of melanoma. The upregulation of mitoribosomes and OXPHOS in melanoma provides a good treatment opportunity for most aggressive and highly proliferative metastatic melanomas.

P048

The context-dependent phenotype of frequently mutated genes: PTEN and KRAS.

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Topic: Cancer

Introduction: A foundation of cancer research includes the identification of frequently mutated genes. PTEN is a tumor suppressor with frequent copy number deletions and normally antagonizes the PI3K/AKT pathway. In contrast, KRAS is an oncogene with common hotspot mutations (G12, G13, Q61). The oncogenic function of KRAS potentially increases pro-growth signaling along the MAPK pathway. While sequencing technologies opened the door to find common mutations in cancer, the consistency of downstream effects at the proteomic and phosphoproteomic levels is unknown.

Methods: Here we analyze the consequence of PTEN loss and KRAS hotspot mutations in nine types of cancer using data from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC).

Results: PTEN loss demonstrates a unique downstream effect in different cancer tissues. The majority of proteins affected by PTEN loss are not shared in multiple cancers, and some cancers have very little affected proteins. Differential protein expression also varies between cancers, including instances of opposite effect. Of the cancers with frequent KRAS hotspot mutations, there is an inconsistent change in the phosphorylation of proteins involved in the MAPK signaling pathway.

Conclusions: The functional consequences of a mutation are generally not consistent across cancers and often have a unique response in different tissues. Further analysis to understand the cancer-specific effect of mutations will improve targeted therapy for individual cancers.

P049

Proteome profiling of MCF-7 and MDA-MB-231 breast cancer cells exposed to genistein, daidzein and a soy seed extract

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Topic: Cancer

Introduction
Breast cancer is the most diagnosed cancer type in women worldwide, its tumour heterogeneity challenging both prognosis and therapeutic strategies. Soy isoflavones have sparked increased interest due to their capacity to interfere in multiple cell signalling pathways. We designed a profiling study to compare the alterations in the proteomes of two breast cancer cell lines, in response to genistein, daidzein, and a soy seed extract (SSE).
Methods
Giving that isoflavones exert dose-dependent effects in estrogen-receptor (ER)-positive cells, MCF-7 cells were exposed to concentrations of test compounds which led to a 20% inhibition of cell growth compared to control (IC20) and to a 20% stimulation compared to control (SC20), respectively. For MDA-MB-231 cells (ER-negative), only the IC20 concentrations were applied. All samples were prepared by using state of the art paramagnetic beads protocol and peptides were subjected to a label-free nano-LC-UDMSE data independent proteomics approach.
Results
In both cell lines SC and IC of the test compounds significantly induced abundance changes for only a small number of proteins (3-4% of all proteins). Hence, in response to daidzein and SSE 50-70 proteins were affected with an equal effect distribution. In contrast, IC20 of genistein impacted more proteins in the MDA-MB-231 cell line (52) in comparison to MCF-7 (15). Most of the altered proteins in the MCF-7 cells displayed an increased abundance. For MCF-7 cell line IC20 upregulated same pathways (cellular component organization, supramolecular fiber organization), genistein and SSE downregulated proteins involved in Tyrosine-Receptor pathway. SC20 of daidzein and genistein affected pathways involved in cell cycle, growth, and differentiation. For MDA-MB-231 cell line, the inhibition following treatment occurred by DNA damage.
Conclusions
Soy isoflavone treatment significantly affected both cell line proteomes with impact on apoptosis and cellular organization. These findings need to be considered essential when investigating soy isoflavones as anticancer agents.

P050

Identification of protein pathways involved in carcinogenesis of breast cancer using LC-MS/MS

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Topic: Cancer

Background: Breast cancer is the fifth most prevalent cause of death among women worldwide. It is also one of the most common types of cancer among Malaysian women. This study aimed to characterize and differentiate the proteome profiles of different stages of breast cancer and its matched adjacent normal tissues in Malaysian breast cancer patients. Also, this study aimed to construct a pertinent protein pathway involved in each stage of cancer.

Methods: In total, 80 samples of tumour and matched adjacent normal tissues were collected. The extracted proteins were fractionated and then analyzed by LC-MS/MS. This study identified the proteins contained within the tissue samples using de novo sequencing and database matching via PEAKS Studio. Two different pathway analyses were performed using DAVID and STRING tools, generate a list of molecules using REACTOME-FI, and added linker nodes in order to develop a connected network. Then, pathway enrichment was obtained, and a graphical model was created to depict the participation of the input proteins as well as the linker nodes.

Results: This study identified 12 proteins that were detected in stage 2 tumour tissues, and 17 proteins that were detected in stage 3 tumour tissues, related to their normal counterparts. It also identified some proteins that were present in stage 2 but not stage 3 and vice versa. Based on these results, this study clarified unique proteins pathways involved in carcinogenesis within stage 2 and stage 3 breast cancers.

Conclusions: This study provided some useful insights about the proteins associated with breast cancer carcinogenesis. Data of the present study provided an improved understanding of the signalling pathways that are implicated in breast cancer. Furthermore, we found sufficient involvement of the Hippo signalling pathway that we recommend additional analysis of its mechanism in carcinogenesis.

P051

Proteasome Footprinting Reveals the Active Degradome of NSCLC in Peptide Resolution

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Topic: Cancer

Introduction:
The mammalian proteasome is estimated to cleave ~70% of all intracellular proteins and is increasingly recognized as a dynamic complex that modulates cellular function in health and disease. However, the regulatory principles targeting specific substrates to proteasomal degradation and their cleavage products are still poorly understood. To enable direct analysis of naturally cleaved proteasomal peptides under physiological conditions, we developed Mass spectrometry Analysis of Proteolytic Peptides (MAPP), a method for proteasome footprinting that allows capture, isolation, and analysis of proteasome-cleaved peptides.
Methods:
To verify whether the isolated peptides were indeed products of proteasomal cleavage, we compared peptides that were identified upon proteasome inhibition to those identified in untreated cells. Likewise, we evaluated whether we could detect the degradation of ZsProSensor, an expected proteasome substrate, using MAPP. Further, we demonstrate analysis of clinical samples by studying tumors from patients with Non-Small Cell Lung Carcinoma (NSCLC).
Results:
MAPP retains information on cleavage patterns of proteasomal peptides as the peptides identified had the canonical proteasome cleavage motifs. When examining the rapid changes in the degradome following stimulation with the inflammatory cytokines TNFα and IFNγ we found 8 proteins known to be involved in the cellular response to inflammation are differentially degraded, including HIF1A, ZFAND5 and CNBP. Finally, we find increased degradation in NSCLC tumors compared to adjacent tissue as well as particularly increased nuclear degradation. The degradation targets found predominantly in tumors represent potential cancer-associated antigens.
Conclusion:
Taken together, MAPP offers a broadly applicable method to facilitate the study of the cellular degradation landscape in various cellular conditions and diseases involving changes in proteasomal degradation, including protein aggregation diseases, autoimmunity, and cancer. The ability to use MAPP in clinical settings with small sample quantities promised to push the forefront of personalized medicine, highlighting novel targets that are crucial to disease pathogenesis.

P052

The landscape of the SKBR3 breast cancer surfaceome and its impact on cancer proliferation

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Topic: Cancer

Introduction: Breast cancer causes the most deaths among cancer diseases besides lung cancer, and HER2+ type cancers are among ones with the worst prognosis. Despite the availability of targeted therapies against the HER2 receptor, the development of drug resistance enables alternate signaling pathways that lead to tumor progression. The present study was aimed at characterizing the cell surface proteome of SKBR3/HER2+ cancer cells by using orthogonal approaches for cell surface protein isolation and mass spectrometry detection.

Methods: SKBR3 cells were cultured in the presence or absence of serum. The cell surface fraction was isolated through the biotinylation of primary amines, biotinylation of oxidized glycans, and enzymatic shaving of the cell surface proteins in the culture. The samples were analyzed by LC-MS using a QE-Orbitrap instrument and data dependent acquisition. Only medium/high confidence proteins (FDR< 3%) with at least two unique peptides were considered for downstream proteome characterization.

Results: The cell-surface protein enrichment protocols were complementary and doubled the number of detectable membrane proteins in the dataset (80 %) when compared to whole-cell proteomic profiling methods (~40 %). Over 1000 cell-surface proteins were detected, with hundreds being differentially expressed. Surfaceome characterization was achieved through a database of ~7000 proteins built in-house based on information from curated databases and literature. Many of the identified cell-surface receptors (RTKs, GPCRs, ion channels, and cell adhesion molecules) were involved in proliferative signaling pathways and/or were reported as being tumor markers. Detected integrins and ADAM family members were found to have a role in altering the expression of HER2 receptors, transactivating EGFR, and offering alternate pathways for proliferation.

Conclusions: Many of the SKBR3 cell surface proteins are part of biological pathways representative of the cancer hallmarks. Their involvement in signaling cross-talk will support discovery efforts focused on identifying targets for more effective disease treatment.

P053

Identification of proteome profile and its signaling networks in pancreatic cancer tissues

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Topic: Cancer

Introduction: Pancreatic cancer, as a highly malignant cancer and the fourth cause of cancer-related death in the world, is one of the most aggressive malignancies with an increase in incidence predicted. Pancreatic cancer is considered a silent disease with poor prognosis and a lack of early biomarkers for detection. A contributory factor to the poor outcome is the lack of appropriate sensitive and specific biomarkers for early diagnosis. Furthermore, biomarkers for targeting, directing and assessing therapeutic intervention, as well as for detection of residual or recurrent cancer are also needed. Thus, the identification of key biomarkers in pancreatic cancer has a critical role and importance for pancreatic cancer treatment. For discovering biomarkers, we focused on key proteins as biomarkers regulated in the expression on neoplastic parenchyma of pancreatic cancer patients-derived tissues compared to normal parenchyma using proteomics technology.

Methods: Proteins for proteomics analysis were extracted and collected from laser captured microdissection (LCM) from pancreatic cancer tissues. From liquid chromatography mass spectrometry-based label-free quantitative protein analysis (LC-MS/MS), data acquisition was evaluated with data-dependent acquisition (DDA) methods in tissue samples from neoplastic and normal parenchyma from patient-derived pancreas cancer tissues.

Results: The protein profiles of neoplastic parenchyma are similar but differed with the protein profiles at normal pancreatic parenchyma. Especially, proteome network from the profiles showed remarkable clusters of among proteins that normal pancreatic function are down-regulated in neoplastic parenchyma.
Conclusions: From our study, understanding the role of protein profiles and their mechanistic action will give insights into the involvement with discovering novel biomarkers as key regulators in pancreatic cancer.

P054

Desmocollin-1 plays a role in breast cancer cell migration, invasion and metastasis and is modulated by parthenolide

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Topic: Cancer

Introduction: Desmocollin-1 (DSC1) is a desmosomal protein playing a role in cell-cell adhesion and tight cell junctions. Recently [1] we identified increased levels of DSC1 in lymph node positive vs. negative primary luminal A tumors. Therefore, we focused on the DSC1 role in molecular mechanisms of lymph node metastasis in luminal A breast cancer and its possible therapeutic modulation.

Methods: A stably transduced, DSC1 overexpressing luminal A breast cancer cell line (MCF7-DSC1-GFP) was generated. Transwell assay and atomic force microscopy were used to study effect of DSC1 overexpression on MCF7 cell migration, invasion, and morphology. Effect of potential DSC1 inhibitors selected in Gene Set Enrichment Analysis (GSEA) of mRNA microarray data set of 341 luminal A tumors was analyzed using western blots and flow cytometry. Quantitative total proteome analysis and pull-down assays were performed in direct data independent acquisition mode on Orbitrap Fusion Lumos mass spectrometer with data analysis in Spectronaut and GSEA.

Results: DSC1 overexpression increased migration and invasion capacity, decreased height of MCF7 cells and led to enrichment of proteins involved in cell cycle regulation, including CDK2, MCM2-7, IBP5 and LACRT in the total proteome analysis ($p<0.05$). Parthenolide, the best performing inhibitor, decreased DSC1 protein levels in MCF7-DSC1-GFP cells, directed them to apoptosis and decreased levels of cell cycle-associated proteins. DSC1 interaction partners included cadherins, protocadherins and tyrosine kinases ERBB2 and ERBB3. Of these, DSC1-ERBB3 interaction was affected by parthenolide ($p<0.05$).

Conclusions: Our systems biology data indicate that DSC1 is connected to cell migration, invasion and cell cycle regulation in luminal A breast cancer cells, and can be effectively modulated by parthenolide, directing the cells to apoptosis.
This work was supported by the Ministry of Health of the Czech Republic, grant No. NV19-08-00250.

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P055

Improved Unified Access to Cancer Proteogenomic Data

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Topic: Cancer

Introduction: Large consortia like the Clinical Proteomic Tumor Analysis Consortium (CPTAC) create comprehensive cancer proteogenomic datasets that are of great value to the scientific community. To meet the broad audience of interested researchers, it is important to disseminate both raw instrument data and fully processed data tables. Unfortunately, processed data tables are usually only distributed as supplemental tables in a manuscript, so there is no standard protocol ensuring convenient access for reanalysis. We present a new and improved version of a data API that provides unified access to processed data tables from nine CPTAC cancer datasets, allowing users to directly stream the latest data to their local environment for analysis.

Methods: We store the data tables in a remote location, organized by data version. We then provide a free Python package that provides access to these tables within the Python programming environment. The package parses the data tables and gives them to the user as dataframes ready to be plugged into statistical, plotting, and other analytical routines.

Results: Our API allows users to easily download the datasets they're interested in and directly access them in their programming environment. The tables follow a simple, consistent format for all cancer types. Since our initial release a year ago, the API has expanded from including three cancer types to nine, and includes new data manipulation utilities. We have also expanded the training material, which introduces common analysis techniques for cancer proteomics so that all audiences can easily understand and use this resource.

Conclusions: Our new, expanded software enables much broader use of the CPTAC datasets by making it simple for anyone to access the finalized data tables. This encourages future reanalysis and new discoveries, and facilitates data reproducibility. Our software model provides a guide for effective data sharing in all areas of science.

P056

Developing an Effective Proteomic Workflow for the Analysis of CAR T-Cells Therapies

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Topic: Cancer

Introduction. Chimeric antigen receptor (CARs) T-cell therapies are an emerging anti-cancer treatment, whereby the patient's immune cells are engineered to express an antigen-receptor targeting tumors of interest. The two FDA approved CAR T-cell therapies, Kymriah and Yescarta, are so far last resort solutions. Wider use of this therapy necessitates innovative approaches like mass spectrometry (MS)-based proteomic analysis to characterize raw material quality attributes (MQA) and final product quality attributes (PQA). Here, we present the development of a MS-based proteomic measurement using data-independent acquisition (SWATH®) to characterize CAR T-cells.

Methods. MS measurements were performed on a TripleTOF® 6600+ LC-MS/MS system (SCIEX). Proteins from Jurkat cells were extracted and digested following four different protocols described below. Peptides were sequenced in data-dependent acquisition (DDA) and quantified with SWATH®. Protein identities from DDA and SWATH® experiments are reported with 1%.

Results. Using Jurkat engineered to express an anti Her-2 CAR on their surface, we are revising the protein extraction and enzymatic digestion to maximize the identified proteome coverage. We are comparing three solid phase assisted approaches – iST, S-Trap, and SP3 – and benchmarking them to the more classical in-solution digestion approach. DDA is performed to create the list of PQPs. We expect the complementarity of the different preparation methods to benefit the proteome coverage. Secondly, SWATH® is applied to collect quantitative data. Evaluation of the extraction methods is based on the following criteria: Number of peptides and proteins identified; Number of missed cleavages; Sequence coverage of the CAR; Identification of known important proteins.

Conclusions. These data will present a streamlined method for the analysis of CAR-engineered cells that will be applicable for the biopharma industry to identify the MQAs and PQAs of CAR-T cells. Moreover, this method can be employed to provide insight on the effect that genetic engineering has on cell biology.

P057

Proteomics profiling of colorectal cancer tissue-derived exosomes reveals non-invasive candidate markers involved in tumor proliferation and progression

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Topic: Cancer

Introduction: Exosomes are small extracellular vesicles (EVs) that are secreted by most cell types to modulate intercellular communication. Cancer derived EVs can enhance tumor progression and metastasis and are detectable in biofluids, thereby providing a liquid biopsy of high interest for biomarker discovery. Most cancer EV proteomics to date has analyzed cell line-derived EVs. Here we focus on patient-tumor tissue-derived EVs as a more relevant sample source to uncover EV functions in cancer and to identify candidate biomarkers with potential non-invasive diagnostics.

Methods: Mass spectrometry-based proteomics was performed on EV fractions isolated using Vn96 peptide from a unique set of 44 clinical samples comprising matched normal and colorectal cancer tissues (n=18) as well as adenomas (n=4). In parallel, tissue and soluble secretome samples were profiled as well. Data analysis was performed using MaxQuant, beta binomial test, GO mining and Cytoscape.

Results: So far we have focused our analysis on the EV fraction of our CRC dataset. In total, 6320 EV proteins were identified, including 3335 proteins with mean count >1.5. Comparative analysis revealed 455 proteins with increased and 182 with decreased abundance in cancer EVs ($p<0.01$; FC>3). Candidate markers with potential for early detection were differential in both normal versus adenoma and normal versus cancer comparisons, yielding 172 proteins involved in RNA splicing, DNA repair and replication, and translation. Furthermore, 64 proteins exhibited a trend profile of increasing/decreasing abundance in control, adenoma, early-, and advanced stage groups. These candidate progression markers were involved in gene expression and translational elongation. Annotation with external datasets yielded 26 unidirectionally deregulated proteins in biofluids (blood, stool, urine).

Conclusions: We report a unique clinical dataset of cancer tissue and EV proteome. Cancer EVs are a reservoir of cancer biomarkers with more notable deregulated proteins compared to tissue. These findings warrant targeted follow-up studies in biofluids.

P058

Identification of autoantigens with diagnostic ability of colorectal cancer by immunoprecipitation coupled to mass spectrometry.

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Topic: Cancer

Introduction and objective

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death. Its five years survival rate depends on the stage of the disease at diagnosis, ranging from 90% for stage I and less than 10% for stage IV patients. CRC detection at early stages can allow the successfully treatment of patients. As the presence of autoantibodies against specific CRC autoantigens in sera of patients has been largely demonstrated, we here aimed to identify autoantigens target of CRC specific autoantibodies with significant ability to discriminate CRC patients from controls by liquid biopsy.

Methods

Three immunoprecipitation assays were performed using as protein sources tissue samples from CRC patients at stages I to IV, and exosomes secreted by 5 CRC cell lines with different metastatic abilities. First, seroreactive proteins to IgGs from CRC patients and healthy individuals were immunoprecipitated. Antigens target of autoantibodies were lately identified by mass spectrometry using a Q-Exactive. Then, their seroreactivity was validated by immunoassays using a higher cohort of serum samples from CRC patients and controls.

Result and discussion

Among the 889 proteins identified, 119 were specifically recognized by IgGs from CRC patients. After bioinformatic analysis, we found that these proteins had been previously described as altered in CRC. Among them, fourteen proteins more prone to be actual CRC autoantigens were selected to analyze their seroreactivity. Finally, a high seroreactivity to CRC patients for some of the autoantigens identified with diagnostic ability of the disease was found.

Conclusion

By immunoprecipitation coupled to mass spectrometry, autoantigens specific of CRC have been identified using different protein sources. Interestingly, we found an enrichment of CRC autoantigens in exosomes, so they could be implicated in their release and should be further investigated to identify cancer specific neoantigens with diagnostic ability.

P059

Multi-omics analyses of Mixed-lineage Acute Leukemia (MLL) focusing on the importance of glycosylation – a pilot study

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Topic: Cancer

Introduction
Mixed-lineage Acute Leukemia (MLL) is one of the most aggressive forms of pediatric cancer. Although the long-term survival rates of Leukemia have increased over the past 40 year, current chemotherapeutic treatment schemes often fail in the treatment of MLL. Therefore, there is an urgent need to identify novel diagnostic and therapeutical targets. Changes in glycosylation have previously been linked to MLL pathogenesis and to the development of MLL drug resistance. We have established the first integrated multi-omics investigation of patient-isolated MLL cells and bone marrow (BM) cells from healthy donors, mapping their proteome, transcriptome and glycome.

Methods
4-6 million cells of 3 normal BM and 2 MLL patients were isolated and analysed by our multi-omics platform. Porous Graphitised Carbon (PGC) nanoLC-ESI-MS/MS was used for Glycomics analyses after the enzymatic and chemical release of N- and O-glycans, respectively. The proteome was explored using RP-LC-ESI-MS/MS analyses, performed after off-line high-pH fractionation, in addition to the RNA-seq analyses.

Results
Overall, 4225 proteins were identified across the patient MLL and control BM cells, of which 216 were overexpressed in MLL ($p<0.01$). Preliminary analyses of the RNA-seq data matches with the proteomics findings, revealing significant alteration in glycoprotein signalling receptors and extracellular matrix proteins, next to various transcription factors. High-pH fractionation allowed us to identify numerous important glycosyltransferases and expression changes thereof on protein and RNA-seq level. N- and O-glycomics revealed overall lower levels of α2-6 sialylated N-glycans in MLL cells (relatable to the level of ST6Gal1 transcript and ST6Gal1 level) and an increase in Core 2 type O-glycans.

Conclusions
Patient isolated MLL cells exhibited distinct N- and O-glycan fingerprints, along with specific alterations of the receptor tyrosine kinase signaling network. In addition to well-known MLL glycoprotein markers, our integrated multi-omics workflow identified a number of previously not described diagnostic/therapeutic protein candidates.

P060

The context-dependent phenotype for oncogenes EGFR and PIK3CA

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Topic: Cancer

Introduction: Previous cancer research identified EGFR (ErbB1/HER1) and PIK3CA as two commonly mutated oncogenes, allowing us to now explore the role of their mutations in cancer development. EGFR is a receptor tyrosine kinase that activates the RAS, MAPK, and AKT-PI3K-mTOR pathways leading to pro-oncogenic signals such as cell growth, angiogenesis, adhesion, and metastasis. Within the EGFR pathway, PIK3CA phosphorylates the lipid PIP2 to form PIP3 and activates the AKT pathway. Both genes have become targets for therapeutic development and precision medicine. Therefore, it is essential to understand their functional consequences. Specifically, it is important to determine whether mutations' effect on the proteome and phosphoproteome are the same across cancer tissue types.

Methods: Here we analyze the consequence of EGFR copy number amplification and PIK3CA hotspot mutations in nine types of cancer using proteomic and phosphoproteomic data from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC).

Results: We show that the vast majority of downstream protein and pathway effects are not observed consistently across cancer types. In fact, EGFR amplification can have opposite proteomic effects on hemostatic proteins depending on cancer tissue type. Additionally, for some cancer types we find no phenotypic effects on the proteomic or phosphoproteomic level.

Conclusion: There is no consistent phenotypic effect of EGFR amplification or PIK3CA hotspot mutation, highlighting the need for cancer specific mutation analysis to enable effective therapeutic development.

P061

Diagnostics of pleural effusions etiology using mass spectrometry-based targeted proteomics

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Topic: Cancer

Introduction: Pleural effusion (PE) is the excess fluid that accumulates in the pleural space. Many diseases may cause PEs, the most common being heart failure, malignancy, pneumonia and tuberculosis. About 50% of lung cancer patients will develop PEs. Determining the etiology of a PE in a quick and non-invasive manner is currently a challenge. A definite diagnosis of a malignant PE requires highly-invasive procedures that collect tissue for histopathological examination. A quick diagnosis is extremely important for patients with benign ailments to avoid unnecessary highly-invasive examinations. The aim of our study is to develop a mass spectrometry-based targeted proteomics assay testing PE samples as a less-invasive, supportive diagnostic tool which effectively discriminates malignant and tuberculous PEs.

Methods: Our developed panel consists of 36 proteins, among them are lung cancer and tuberculosis biomarkers already used in the clinics as well as potential biomarkers published in the literature and characterized by high specificity and sensitivity. The great majority of the biomarkers are present at low levels even at pathological states. The sample preparation method was optimized and a Multiple Reaction Monitoring (MRM) assay was developed with 107 stable isotope-labeled internal standard (SIS) peptides (13C,15N-C-term-K/R). 211 pleural fluid samples were collected from patients of the Mazovian Centre for Treatment of Lung Diseases and Tuberculosis and analyzed by nanoflow liquid chromatography coupled with tandem mass spectrometry (nano-LC-MS/MS).

Results: 31 from 36 proteins of interest were detected. Five proteins discriminate tuberculous PEs from other groups with statistical significance $p<0.0001$. Overexpression of Mesothelin was observed in all PEs caused by mesotheliomas and in a part of non-small cell lung carcinoma PEs but was absent in small cell lung carcinoma PEs.

Conclusions: The developed panel potentially could be useful in tuberculous PE identification. A classifier tool is being built that can predict PE etiology.

P062

Mass spectrometry based profiling of histone post-translational modifications for identification of novel biomarkers in TNBC and patients stratification

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Topic: Cancer

Introduction: Triple Negative Breast cancer (TNBC) is the most aggressive form of breast cancer and is associated with poor prognosis. Four main subtypes of breast cancer have been identified (Luminal A, Luminal B, HER2+, and TNBC) depending on their molecular features and TNBC is the only subtype for which no molecular markers have been identified and no specific therapies other than chemotherapy are currently available. For these reasons, a better understanding of the molecular mechanism of this disease should be investigated with the purpose to identify novel biomarkers for patients stratification, and development of new targeted therapies. Aberrations in histone post-translational modifications (PTMs) have been reported as hallmarks of cancer and as prognostic markers. Thus, in this study we employed a quantitative mass spectrometry (MS) approach for the analysis of histone PTMs to profile epigenetic changes in the different breast cancer subtypes, focusing mainly on the characterization of Tumor vs Normal and TNBC vs Luminal-like subtypes.

Methods: We profiled breast cancer patient samples using a LC-MS/MS method which allows the evaluation of histone PTM patterns in formalin fixed paraffin embedded tissues (FFPE), OCT frozen tissues, and fresh frozen samples, in combination with a histone-focused super-SILAC approach. To further identify novel biomarkers we are currently intersecting data from MS analysis with ChIP-seq to identify potential epigenetic mechanisms.

Results: The MS analysis performed on >100 breast cancer samples revealed epigenetic signatures that distinguish the different subtypes. More specifically H3K4me2, H3K9me3, and H4K20me3 marks have been selected as potential biomarkers in TNBC.

Conclusion: The histone patterns identified so far offer insights into potential epigenetic mechanisms underlying breast cancer, and particularly TNBC, not only providing biomarkers useful for patient stratification but also suggesting novel epigenetic pathways targetable for therapy.

P063

Refinement of targeted-MS parameters tailored for the detection of ultra-low abundant neoepitopes

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Topic: Cancer

Direct detection of tumor-derived neoepitopes, in the context of personalized immunotherapy, poses an analytical challenge as neoepitopes can be of very low abundance. Therefore, currently several hundred million cells are required for their detection, which is in direct conflict with the usually limited amounts of available patient samples.

Orbitrap instrumentations coupled to the new Field Asymmetric Ion Mobility Spectrometry (FAIMS) interface could increase the sensitivity required for rare peptide detection. However, it entails fine-tuning of essential instrument parameters. This differs from the common practice that, despite the chemical diversity of peptides, default Orbitrap parameters, or at best normalized to mass-to-charge values, are often used without further consideration.

Aiming at reaching ultrahigh sensitivity, we implemented an efficient procedure for optimizing various detection parameters using static spray instead of the common LC-MS methodology. Our approach is preventing lengthy steps of optimizations including multiple back-and-fourth stages wending through multiple software platforms. Although our approach is applicable to the fine-tuning of any parameter, we show its utility for NCE, RF lens and peptide-specific compensation voltage (CV) as part of the FAIMS interface. A bioinformatics application is under development so that this procedure will be easily accessible to the researchers in the field.

As demonstrated for the detection of low abundance HPV16-derived HLA-A2-restricted peptides, our results show an increase in sensitivity of a PRM assay by several orders of magnitude. The benefit of the FAIMS interface is especially visible in the case of immune-isolated HLA-A2-restricted peptides with a particularly high background of small proteins. The ultimate aim is to reach high sensitivity for peptide detection without excessive tumor cell expansion that inherently involve the risk of clonal divergence. The resulting accuracy and speed of the analysis will finally benefit tumor patients.

P064

Proteomic profiles of small cell lung cancer cell lines: Correlation with molecular subtypes and in vitro growth characteristics.

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Topic: Cancer

Introduction: Small cell lung cancer (SCLC), an especially aggressive malignancy, is characterized by rapid growth and early metastasis. However, no substantial improvement has been achieved in its therapy during the last three decades. Recent genomic studies suggest that SCLC has distinctive subtypes, based on the relative expression of four key transcription factors (ASCL1, NEUROD1, YAP1 and POU2F3) [1]. Our aim was thus to further characterize these four SCLC molecular subtypes at the proteome level, and to identify novel subtype-specific diagnostic and prognostic markers.
Methods: 19 patient-derived cell lines were grown and characterized by describing their molecular subtypes (as defined in Rudin et al.) and in vitro growth patterns (i.e. adherent or suspension cells). The harvested cells were then subjected to MS-based shotgun proteomic analysis using label-free quantification, followed by data quality assessment and statistical evaluation in order to identify fundamental biological processes and pathways.
Results: The in-depth proteomic analysis identified more than 9000 proteins. High quality data was obtained, indicated by the high overlap of the identified proteins between the cell lines. Clustering analysis confirmed the presence of the aforementioned four subgroups. Our data suggests that both the molecular subtype and the in vitro growth characteristics had a significant influence on the proteomic profiles.
Conclusions: Our proteomic study supports the concept that SCLC is a heterogeneous disease. We plan to extend our investigation by including additional cell lines and by secretome analysis. We believe that the results will supplement the current information on SCLC heterogeneity, based primarily on genomics, and will contribute to the development of a new SCLC classification.
[1] Rudin CM, et al. Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data. Nat Rev Cancer. 2019;19(5):289-97.

P065

Global Proteomics and Phosphoproteomics Analysis of Formalin-Fixed Paraffin-Embedded and Frozen Tissue Sections reveal Intra-tumor heterogeneity in Malignant Melanoma

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Topic: Cancer

Introduction: Malignant Melanoma (MM) is the main cause of death related to skin cancer. Formalin-fixed, paraffin-embedded (FFPE) tumor tissue specimens offer an invaluable resource for large-scale studies aimed at discovering new clinical biomarkers. Here we performed deep proteomic and phosphoproteomic characterization of FFPE and fresh frozen melanoma tumors (FFT) samples and evaluates the signaling pathways related to intra-tumor heterogeneity (ITH).

Methods: FFPE (n=30) and FFT (n=20) tissues were collected from primary tumors and lymph node metastases of 10 patients diagnosed with MM. FFPE samples were deparaffinized with EnVision Agillent solution (1:50) at 97˚C. For protein extraction, the samples were incubated in an extraction buffer (25mM DTT, 10 w/v% SDS, 100mM TEAB pH 8.0) at 99˚C for 1 hour in the FFPE and for 5 min in the FFT, followed by 40 cycles, 15s on/off in the Bioruptor. The S-Trap was used for sample digestion using LysC (1:50, 2 hours, 37 °C) and trypsin (1:50, 37 °C, overnight). One-microgram of peptides was injected in the UltiMate-3000 nanoLC coupled to the Q Exactive HF-X. Mass spectra were analyzed in Proteome Discoverer 2.4, and the statistical analysis was made in the Perseus software.

Results: We identified 7200 protein groups, 13223 phosphopeptides, and 14624 phosphosites across all the samples. The Pearson correlation coefficient in the quantitative label-free analysis shows a high correlation between FFPE and FFT samples, with a value of 0.94 for the total proteome and 0.74 for the phosphopeptide analysis. The main signaling pathway related to ITH, are linked to TP53 regulation, BRAF signaling, and antigen processing.

Conclusions: The comparison between FFPE and FFT revealed that main signaling pathways are preserved, even at the phosphoproteome level. Proteins involved in ITH are crucial to deep-meaning cancer progression, therapy resistance, and recurrences.

P066

Proteomics reveals heterogeneity in the glioblastoma microenvironment

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Topic: Cancer

Introduction

Glioblastoma (GBM) is the most common and aggressive malignant brain tumor, with a median survival of only 14.6 months after treatment with surgery, radiation, and chemotherapy. Genome and transcriptome profiling have revealed a complex and evolving landscape of aberrations, but it remains unclear how these alterations affect the proteome and whether they converge on a limited set of functional states that promote tumor growth and resistance to therapy. Furthermore, the presence of clinically significant heterogeneity in the tumor microenvironment (TME) of GBM means that current single-sample profiling strategies may miss TME-dependent aspects of tumor evolution. Here we address these challenges through proteomic profiling of multiple spatially distinct regions sampled from GBM tumors both before and after therapy.

Methods

Fresh-frozen tumor samples (n=37) from multiple regions and/or time points of 4 GBM patients were analyzed with fractionated LC-MS/MS and multiplexed using TMT-11 reagents to quantify ~8,200 proteins per array. We performed unsupervised classification using non-negative matrix factorization, and deconvolved latent factors corresponding to sample subgroups. These were further characterized using Gene Set Variation Analysis to define biological themes that varied in space or with therapy.

Results

We identified significant proteomic heterogeneity between regions of individual tumors both before and after therapy. Rather than observing discrete clustering of primary versus relapsed samples, we found strong proteomic similarity between 2 or more subsets of primary and recurrent tumor regions within a patient, indicating a possible functional convergence on a limited set of co-existing proteomic states. Some of these states correspond to distinct TMEs that persist through therapy and include the presence of infiltrating immune cells (e.g. macrophages and neutrophils).

Conclusions

Global quantitative proteomics was used to identify substantial spatial heterogeneity in GBM and a limited set of tumor-immune microenvironments, indicating a common facet of tumor evolution that persists or is re-established post-therapy.

P067

Proteomic differences in early versus late brain metastases development in a cohort of lung adenocarcinoma patients

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Topic: Cancer

Introduction: Lung cancer is the cause of most cancer-related deaths worldwide, with lung adenocarcinoma (LUAD) accounting for 40% of all pulmonary cancers. Half of the patients eventually develop brain metastasis, but the time required for the formation of brain metastasis is unclear. Most patients die within one year of diagnosis and 20% can only receive palliative care. Therefore, it is very important to investigate brain metastasis development in LUAD and search for new therapeutic targets and prognostic markers. In this this study, we characterized FFPE tissue samples of primary tumors and their corresponding brain metastases from 20 LUAD patients using in-depth proteomic analysis supplemented with detailed clinical and histopathological data.

Methods: The FFPE samples were deparaffinized, followed by protein extraction with SDS and digestion using suspension trapping. Peptides were analyzed by nLC-MS/MS (Ultimate 3000 RSLC nano pump coupled to Q-Exactive HF-X) with label-free quantification. Raw data was searched using Proteome Discoverer vs2.4 and bioinformatical analyses were performed in Perseus, RStudio and GraphPad Prism. Histopathological analyses were carried out using QuPath.

Results: In total, 7676 proteins were quantified across all samples. Outlier analysis detected one outlier, where the associated histopathological analysis revealed low tumor content. Unsupervised hierarchical clustering showed that the samples clustered mainly according to their tissue origin. Therefore, primary and metastatic samples were treated separately for further analyses. Student's t-test (p-value 0.05) identified a set of differentially expressed proteins between rapid and slow progressing patients. Pathway analysis revealed interesting pathways associated with rapid and slow progression to brain metastasis, such as EGFR-related pathways in brain metastasis samples with rapid progression.

Conclusions: Our study provides a comprehensive proteomic analysis of lung adenocarcinoma brain metastasis progression in a unique cohort of paired primary and metastatic tumors. We identified proteins and pathways possibly associated with rapid and slow metastatic progression.

P068

Plasma protein profiling reveals low serum paraoxonase 1 levels in DCM patients

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Topic: Cardiovascular and Metabolic Diseases

Introduction
Dilated cardiomyopathy (DCM) is a heart disease frequently leading to heart failure (HF) and a major worldwide health problem. In contrast to other HF causes, knowledge on circulating plasma proteins in DCM patients is still poor. Therefore, this study comparatively analyzed plasma protein profiles of DCM patients and individuals with low normal and normal left ventricular ejection fraction (LVEF) by mass spectrometry.

Methods
The study was performed retrospectively using plasma samples of DCM patients with LVEF ≤ 45% and left ventricular external diameter at diastole (LVEDD) ≥55 mm in comparison to individuals with LVEF ≥ 50%. Global proteome profiling was performed using an LTQ-Orbitrap Velos mass spectrometer.

Results
Differentially abundant proteins are involved in lipid metabolism, coagulation, and acute phase response. Strongest differences were observed for serum paraoxonase 1 (PON1), and were validated by targeted protein analysis in an independent patient cohort. PON1 levels were also determined by an ELISA. Additionally, in a linear regression study including 349 DCM patients adjusted for age, sex, and body mass index a lower PON1 level was observed with decreasing LVEF function. These data highlight PON1 as a potential marker for differentiating DCM patients not only from patients with normal LVEF, but also from heart failure patients with preserved ejection fraction.

Conclusion
The current study reveals that the impaired serum arylesterase activity reported in DCM patients is likely caused by decreased circulating PON1 levels contributing to lipid metabolism alterations and oxidative stress.

P069

Advanced glycation end products (AGE) PTM profiling on antigen processing machinery and MHC-II molecules in diabetes and T2DM syndrome

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Topic: Cardiovascular and Metabolic Diseases

Introduction
Increased oxidized ribose, glucose, glyoxal and methylglyoxal concentrations are observed in several metabolic diseases including diabetes and metabolic syndrome, mediating the non-enzymatic protein glycation and carbonylation followed by protein unfolding, cross-linking and aggregation. We investigated protein modifications induced by glycation and glycoxidation, i.e. the AGE-modified proteome, using peripheral blood mononuclear cells (PBMC) from non-diabetic and diabetic patients. The AGE-amide protein adducts were mapped by bottom-up label-free proteomics employing a QExactive HF quadrupole orbitrap mass spectrometer. Using bioinformatics, we found that several cellular pathways were affected, including endosomal maturation and antigen presentation and processing machinery. Further mapping of PTM on the in vitro glycated human MHC II allowed the identification of key AGE-modified amino acids residues involved in peptide epitope binding.

Methods
We purified PBMC from healthy and diabetic patients and mapped the AGE-PTM by bottom-up proteomic analysis of trypsin/Asp-N/Glu-C/Lys-C digests of total clinical proteomic extracts from PBMC. Glycated peptides were analyzed using HCD MS/MS on a Q Exactive HF quadrupole orbitrap mass spectrometer in data dependent acquisition (DDA) mode.

Results
We mapped a difference in the total number of unique AGE sites (485 versus 22) in the diabetic versus healthy controls. Analysis of the type of AGE-PTM accumulated in the proteomes from “diabetic” samples ranked the site-specific carboxymethylation of lysine (CML) as the most abundant AGE found in the glycated proteome, followed by formylation of lysine and carboxymethylation of arginine (CMA).

Conclusions
Using comprehensive proteomics and bioinformatics tools, we identified that several proteins involved in antigen processing and MHC II peptide/loading were subject to both AGEs and carbonyl modifications mediated by the redox metabolic stress characterizing the T2DM condition. These results highlight the potential role for accumulation of AGE-PTM in the pathophysiology of immune responses.

P070

Diagnosis and functionalization of the mitochondrial disease-associated ATAD3 gene cluster

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Topic: Cardiovascular and Metabolic Diseases

Introduction: Mitochondrial diseases are a heterogenous group of disorders caused by mutations in over 350 known genes, affecting ~1:5000 live births. The high degree of clinical, biochemical and genetic heterogeneity of mitochondrial disorders poses an immense challenge for molecular diagnosis with a significant portion of cases remaining unsolved. The ATAD3 locus is comprised of three highly homologous, tandemly arrayed genes (ATAD3A, ATAD3B, and ATAD3C) and despite being implicated in different cellular mechanisms such as cholesterol metabolism, mitochondrial DNA replication, dynamics and morphology, its precise function remains unclear. The high homology of the locus makes the genomic region prone to a spectrum of pathogenic copy number variations such as intergenic deletions and duplications.
Methods: We developed a quantitative proteomics based approach, that when combined with other genomic data allowed us to identify de novo ATAD3 duplications in a cohort of 17 subjects from 16 unrelated families with cardiomyopathy, persistent hyperlactacidemia, and frequently corneal clouding or cataracts (1).
Results: The duplications all resulted in the formation of an identical chimeric ATAD3A/ATAD3C protein, confirmed by quantitative proteomics, which appears to act in a dominant manner causing altered ATAD3 complexes and a striking reduction in mitochondrial OXPHOS complex I activity in heart tissue. Similarly, patients harboring deletions in the ATAD3 cluster presented with decreased abundance of peptides that match the deleted genetic region in the protein.
Conclusions: Although the ATAD3 locus was previously refractive to study due to the highly repetitive nature of the genomic region, it has now emerged as one of the five most common causes of nuclear-encoded paediatric mitochondrial disease. We are now applying the proteomics techniques we have developed here and elsewhere, along with gene-edited cell lines and patient derived iPSC lineages to functionalize the genes encoded by the ATAD3 locus.

1.Frazier et al. 2020, Med. https://doi.org/10.1016/j.medj.2020.06.004

P072

DIA-MS proteotyping and superresolution imaging for subcellular phenotype and clinical subtype classification of aortic valve stenosis in cardiac patient biopsies

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Topic: Cardiovascular and Metabolic Diseases

Introduction

Severe aortic valve stenosis (AVS) represents the most prevalent ageing-associated cardiovalvular disease, with ~50% mortality within two years. Based on echocardiography, AVS is classified into distinct subtypes I-IV. To elucidate subcellular disease mechanisms, we analyzed left-ventricular (LV) cardiac biopsies from a patient cohort treated by transcatheter aortic valve implantation by 1) DIA-MS, 2) superresolution STED microscopy (nanoscopy), and 3) clinical phenotype correlation.

Methods

For each AVS subtype I-IV, five LV biopsies from individual patients were lysed andtryptically digested using pressure cycling and analyzed by DIA-MS using an in-house spectral library generated from a pooled reference sample. LV tissue from nonfailing donor hearts served as control. Protein abundance profiles were analyzed by unbiased hierarchical clustering and pairwise comparison. In parallel, proteins-of-interest (POIs) were analyzed in fixed LV biopsy sections by multi-color STED nanoscopy.

Results and Discussion

Using a spectral library of 2970 cardiac proteins, we obtained quantitative profiles for 2273 proteins across all LV biopsy samples (n=25). Unbiased hierarchical clustering showed four distinct proteotypes which correlated with the echocardiographic subtypes. Pairwise comparison revealed significant abundance changes for POIS involved in cardiac muscle contraction, cell-cell adhesion, energy metabolism and protein folding. Importantly, the Ryanodine Receptor (RyR2) Ca2+ release channel was significantly downregulated across AVS biopsies. STED nanoscopy revealed subcellular RyR2 cluster fragmentation. Importantly, this strongly correlated with decreased LV ejection fraction in AVS subtypes I-IV.

Conclusions

Combined proteomic profiling and superresolution imaging of LV biopsies from AVS patients identified RyR2 as a disease-relevant POI. While RyR2 expression was significantly decreased, RyR2 cluster fragmentation was significantly increased. As RyR2 is essential for cardiac excitation-contraction coupling, our data suggest previously unknown subcellular mechanisms of contractile dysfunction across AVS subtypes. In situ cardiac proteome profiling and superresolution imaging provided a robust basis for clinical phenotype correlation and molecular subtype analysis.

P073

Stable Isotope Labelling in Mammals (SILAM) and proteomics characterization of individual vascular layers in sepsis-induced acute inflammatory response

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Topic: Cardiovascular and Metabolic Diseases

Introduction: Alterations in whole organism vascular beds and endothelial dysfunction play key roles in organ failure and in triggering different life-threatening diseases. However, the molecular mechanisms of these pathological events remain poorly understood. Thus, we analyzed by systems biology the in vivo proteome dynamics of individual vascular beds, isolating the proteomes of glycocalyx (GC), endothelial cells (ECs) and smooth muscle cells (SMCs) from the vascular system of whole organisms, following the induction of an acute vascular inflammatory challenge.

Methods: In order to label newly synthesized proteins, mice dietary Lys was replaced for its stable isotope Lys(6), as defined by stable isotope labeling in mammals (SILAM) strategy. Generation of the acute endotoxemia model consisted in acute administration of lipopolysaccharide endotoxin intraperitoneally. Subsequently, vascular bed proteomes were selectively decellularized and solubilized by differential systemic decellularization in vivo (DISDIVO). The obtained vascular proteomes were processed and analyzed by unbiased discovery-driven shotgun proteomics.

Results: A drastic reduction in the synthesis of new proteins related to key molecular mechanisms in ECs was observed, which in turn interfered with homeostatic molecular maintenance of the GC. SMCs proteomes showed similar patterns to those identified in the former internal vascular layers though to a lesser extent. Specific proteins, such as haptoglobin, hemopexin and serum amyloid in ECs, as well as S100A9, LGALS1 and some histones in SMCs, showed an increase in protein synthesis. Furthermore, we also observed a significant intensification of ECs protein turnover, and identified unique disease-specific phosphorylation sites in ECs proteomes.

Conclusions: New molecular insights into the inflammatory response of vascular beds were achieved in this study. Understanding the molecular dynamics that precede endothelium dysfunction at the onset of acute sepsis might help to develop novel precision therapeutic strategies.

P074

Quantitative Proteomics and Comprehensive Bioinformatics Provides Insight into the Glucose-Lowering Independent Effects of Antidiabetic Drugs on Coronary Artery Endothelial Cells

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Topic: Cardiovascular and Metabolic Diseases

Introduction: Type 2 Diabetes Mellitus (T2DM) confers an elevated risk of mortality due to cardiovascular disease (CVD). Despite the great repertoire of antidiabetic drugs (ADD) that regulate blood glucose in subjects with T2DM, only a few (e.g., empagliflozin) have demonstrated clear benefits on CVD endpoints, while others are associated with adverse cardiovascular effects (e.g., rosiglitazone). Yet, the direct effects of ADD on coronary endothelium —involved in CVD development— are not known. In this study, we explored the effects of four ADD (including empagliflozin and rosiglitazone) on Human Coronary Artery Endothelial Cells (HCAEC) using quantitative proteomics and bioinformatics.

Methods: HCAEC were treated with clinically relevant concentrations of metformin (100 µM), rosiglitazone (1 µM), empagliflozin (1 µM), saxagliptin (1 µM) or vehicle (DMSO) for 48 hrs. SWATH-based proteomics was used to determine protein abundance differences among groups (ADD vs. vehicle), using a previously established methodology (1). Two different bioinformatics (OmicsNet and ClueGO) approaches were utilized to explore the signaling pathways (REACTOME and KEGG Databases) modulated by the ADD. We further created an integrated protein network between the ADD and well-known inflammatory cytokines (TNFa and IL-1B) to explore the anti-inflammatory effects of the ADD.

Results: Both bioinformatic approaches revealed a down-modulation of signaling pathways linked to inflammation by all ADD, but at different levels. TNFa-triggered activation of p53 and NF-kappaB signaling, while all ADD, excepting saxagliptin, down-modulated proteins involved in p53 signaling (within the top 10 significant pathways). Particularly, saxagliptin and empagliflozin up-modulated proteins linked to mitochondrial oxidative phosphorylation and AMPK signaling, respectively.

Conclusions: ADD modulate signaling pathways associated with inflammation and mitochondrial activity, which may account for their beneficial glucose-lowering independent effects on HCAEC.

1. Delgado De la Herrán., et al. 2019 bioRxiv https://doi.org/10.1101/622407

P075

Towards an optimized heart tissue preparation procedure for comprehensive proteomic analysis

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Topic: Cardiovascular and Metabolic Diseases

Introduction: Sample lysis is one of the most crucial steps in tissue preparation for subsequent proteomic analysis. The quality and reproducibility of sample extraction and preparation significantly affect MS results. Therefore, we aimed to optimize lysis conditions as well as the reduction and alkylation process for mouse heart samples to establish a robust workflow for consistent, high quality results.

Methods: Cell and/or tissue lysis is the first step in protein extraction. Numerous physical and reagent-based methods have been evaluated to obtain the best possible yield for different organisms and sample types like cells or tissue. In this regard, we tested different SDS buffers with mechanical cell disruption methods to circumvent substantial sample prep variabilities. Furthermore, as most common mass spectrometry workflows for complex protein samples analyze peptides, cysteine containing peptides have to be treated to enhance proteolytic digest efficiency and avoid artificial disulfide bond formation using a reducing agent such as Tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) for disulfide reduction. Representing another source of sample variability protocols using different reduction (DTT and TCEP) and alkylation agents (CAA, IAA, and MMTS) were tested.

Results: Here, we established a protocol using a SDS extraction buffer in combination extraction by bioruptor. Additionally, the combination of TCEP/CAA proved to be the most effective reduction/alkylation technique for preparation of mouse heart proteins.

Conclusions: Finally, a complete sample preparation pipeline from lysis to alkylated peptide sample could be established using lysis by highly controlled ultrasonic energy (Bioruptor) combined with a 1% SDS buffer, reduction with TCEP and alkylation via CAA.

P076

Cardiosphere Derived Cell-Induced Cellular Reprogramming of Transcriptional and Translational Machinery in Heart Failure with Preserved Ejection Fraction

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Topic: Cardiovascular and Metabolic Diseases

Introduction:
Heart failure with preserved ejection fraction (HFpEF) is characterized by diastolic dysfunction leading to insufficient filling of the heart due to increased myocardial stiffness. Previous studies showed that treatment with cardiosphere-derived cells (CDCs) improved diastolic function and survival rates in a Dahl salt-sensitive rat model. The focus of this study was to identify the upstream regulators that drive the proteome changes responsible for the underlying CDC responsive genetic pathways.
Methods and Results:
Dahl salt-sensitive rats fed high-salt diet, with echocardiographically verified diastolic dysfunction, were randomly assigned to either intracoronary CDCs or placebo. Dahl rats receiving low salt diet served as controls. CDC treatment decreased diastolic dysfunction indicated by a normalized E/A ratio, a 33.3% reduction in Tau, and a 47% reduction of LVEDP. Total protein, isoforms and phosphorylation status of left ventricular tissues (n=6/group) were quantified by mass spectrometry. The proteomic data revealed dominant changes in proteins involved regulating protein synthesis accounting for 37% protein and 19% protein phosphorylation changes. Upstream regulator analysis identified that 65% of all CDC therapeutic mediating pathways to be linked to 7 upstream regulators. L-Azidohomoalanine labeling of newly synthesized proteins was carried out in isolated adult cardiomyocytes obtained from HFpEF rats, treated with CDC exosomes or placebo confirming 5 upstream regulators. Western blot analysis showed, that TGFb1, TP53, and Myc were significantly decreased, whereas MTOR and HNF4A showed a significant increase in CDC treated animals. Finally, all 5 upstream regulators could be regulated by proteins or miRNA that were identified to be present in CDC-derived exosomes as cargo.
Conclusion:
We identified 5 potential key regulators mediating CDC therapeutic myocardial response by targeting the cellular transcription and translational machinery and linked these to regulator proteins and miRNAs within the exosome cargo.

P077

In-Depth Proteomic Characterisation of Different Aetiologies of Cardiomyopathy

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Topic: Cardiovascular and Metabolic Diseases

Background

The purpose of this study was to gain greater understanding of the pathogenesis of hypertrophic obstructive cardiomyopathy (HOCM), dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ISCM). These conditions lead to heart failure (HF) and the prognosis for HF differs based on the underlying aetiology. Cardiac tissue represents a challenging sample from the proteomics perspective due to the dominant signal from of a small number of high abundance proteins. Thus, a diaPASEF workflow was applied in order to achieve deep quantitative coverage of cardiac tissue from HOCM (n=12), DCM (n=9), ISCM (n=9) and healthy controls (n=9).

Methods

Unbiased, deep proteomic analysis of individual samples was performed using the diaPASEF workflow on a timsTOF Pro mass spectrometer. Analysis of high pH-reversed phase fractionated sample pools was performed in ddaPASEF mode to generate spectral library data. Raw data files were processed through Spectronaut 14 software for spectral library building, protein identification and quantification. Further statistical analysis was performed using Perseus (V1.6.2.2).

Results

Label-free MS analysis led to 5,588 protein identifications, with an average of 5,263 proteins identified per sample and 3,851 proteins commonly identified across all patient samples and technical replicates. Analysis of significant up and down-regulated proteins highlighted the Acute Phase Response Signalling pathway as being associated with HOCM and ISCM, whereas the LXR/RXR pathway was found to be most associated with DCM. Differentially expressed proteins unique to HOCM (n=6), DCM (n=2) and ISCM (n=43) were identified based on an observed fold change of ≥ 1.5 or ≤-1.5 and q-value ≤ 0.005.

Conclusions

This represents one of the deepest proteomic datasets for myocardial tissue reported to date, generated from a label-free MS analysis, without need for extensive LC-based pre-fractionation of individual samples. The dataset has highlighted disease-relevant pathways and biomarker candidates. Further evaluation of these findings is ongoing in independent patient cohorts

P078

Elucidating the Senescent Surfaceome for Development of Senolytic Human Therapies

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Topic: Cellular and Spatial Proteomics

Introduction: Cellular senescence has emerged as a promising therapeutic target for a multitude of chronic age-related conditions, ranging from neurodegeneration to cancer. Currently, there are no markers amenable to targeting senescent cells for isolation or therapeutic intervention. Therefore, senescence-specific cell surface markers are needed to develop and translate therapeutic approaches of eliminating senescent cells into humans. Here, we profile the surfaceome of senescent human fibroblasts, experimentally validate several surfaceome targets, and predict surface targets for targeting endogenous senescent cells from human tissues.

Methods: The surfaceomes of human lung fibroblasts (IMR90) were collected using Lysine-Cell-Surface-Capture (Lys-CSC). Lys-CSC uses the cleavable and non-cell-permeable biotinylation reagent Sulfo-NHS-SS-Biotin to biotinylate the primary amines on accessible lysines on the surface of live cells. The cells are harvested, proteins are digested, and peptides derived from cell surface proteins are purified by avidin affinity. Senescence-specific cell surface proteins were determined by comparing the surfaceomes of ionizing radiation (IR)-induced senescent fibroblasts to quiescent fibroblasts. Data-independent acquisitions (DIA) were performed on a TripleTOF 6600 and analyzed in Spectronaut (Biognosys) using the pan-human spectral library.

Results: We identified 59 enriched and 44 decreased proteins on the surface of senescent fibroblasts induced by IR compared with quiescent controls (n=10) and validated three candidates by quantitative flow cytometry. These markers are specific to senescent fibroblasts with variable degrees specificity to tissue types according the Cell Surface Protein Atlas. Senescent surfaceome targets were enriched with pathways involved with cell death and vesicle-mediated trafficking. We also predicted senescent surface protein candidates in multiple subtypes of senescent cells using published transcriptomics in combination with in silico analysis tools.

Discussion: Identification of senescence-specific cell surface markers will provide targets for the delivery of interventions to senescent cells and will lay the groundwork for methods to isolate intact endogenous senescent cells from tissues for the first time.

P079

Dissecting the Ontogenic Remodeling of the Proteomic Landscape and Functionality of Hematopoietic Progenitor Cells in Normal Development and Leukemia

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Topic: Cellular and Spatial Proteomics

Introduction: The process of blood cell development (hematopoiesis) is subject to extensive ontogenic remodeling that is accompanied by alterations in cellular fate both during normal development and disease. Although the functional differences between fetal and adult hematopoiesis are well-established, the responsible molecular mechanisms have remained largely unexplored on the proteomic level. We have previously characterized the proteome of the earliest hematopoietic stem and progenitor cells in the fetus and the adult (Jassinskaja et al., 2017), but the molecular programs that orchestrate functions related to differentiation capacity and lineage-bias during ontogeny and disease remain unresolved. Importantly, lineage-biased hematopoietic progenitor cells represent a critical target for investigation, as such cells can act as potent leukemia-initiating cells.
Methods: We have utilized in-stage tip (iST)-based methods for sample preparation combined with isobaric labeling and an SPS-MS3 approach to gain deep coverage of the proteome of 100,000 fetal and adult lymphomyeloid, lymphoid-biased and myeloid-biased hematopoietic progenitor cells.
Results: Our analysis resulted in the identification and quantification of over 4000 proteins, with 200-300 proteins per cell type displaying differential expression between the fetal and the adult cells. We uncovered ontogenic changes in expression of several transcription factors with critical roles in hematopoiesis. Together with other proteins involved in lineage commitment and differentiation, we show that protein expression in fetal and adult hematopoietic progenitor cells is predictive of differential lineage potential. Our subsequent functional analyses further emphasize these differences, which are evident both in the context of normal differentiation and leukemic hematopoiesis driven by the MLL-ENL fusion oncogene.
Conclusions: Collectively, our work represents a significant advancement in the understanding of the molecular programs that govern ontogenic differences in hematopoiesis and provides a solid foundation for future investigation of which factors are responsible for the difference in susceptibility and outcome of different leukemias in infants and in adults.

P080

Leveraging computational approaches to explore protein interaction dynamics and localizations across space and time

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Topic: Cellular and Spatial Proteomics

Introduction: An inherent benefit of proteomic analyses is the capacity to yield multifaceted datasets that span subcellular compartments and biological pathways. Combined with recent technological advancements, researchers can now regularly generate proteomes with increasing breadth and depth. Yet, a fundamental barrier when analyzing increasingly intricate datasets is the lack of tools to extract biologically significant targets from the study population. As a result, there is a growing demand for pipelines that facilitate data analysis and integration to reveal both individual proteins of interest and the dynamics of protein regulation at a global level.

Methods: To address this issue, we have developed computational platforms called TRANSPIRE (1) and Inter-ViSTA (2) that augment the analysis of spatial proteomics and protein-protein interaction datasets, respectively. To demonstrate their utility, we applied these tools to study protein localization and interaction dynamics across space and time during infection with human cytomegalovirus (HCMV), a pathogen that broadly reorganizes the host proteome.

Results: Analysis with TRANSPIRE confirmed that HCMV regulates protein dynamics at a global scale and further revealed that infection stimulates previously unappreciated, widespread changes in protein localization. Notably, we identified that HCMV redistributes proteins associated with cellular processes that are known to impact viral replication, including host defense, metabolism, and cellular trafficking. Furthermore, Inter-ViSTA analysis of interactions with the viral protein pUL37 revealed temporally-distinct roles for this protein in remodeling mitochondria and peroxisomes throughout infection.

Conclusions: Discoveries prompted by TRANSPIRE and Inter-ViSTA reinforced the multifaceted nature of HCMV-induced proteome alterations. Together, these platforms augmented our understanding of HCMV biology, further elucidating host and viral protein dynamics across space and time.

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P081

Proteomics in In Vitro Toxicity Screening of Amorphous Silica Nanoforms

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Topic: Cellular and Spatial Proteomics

Background: Nano-sized amorphous silica are used in various applications including consumer products, engineering and medical technologies enhancing the potential for exposure. Attractive physicochemical properties of these nanomaterials (e.g. smaller size, larger surface area) enhance their usage and thus increase the likelihood for environmental/human exposures. Risk assessment of silica nanoparticles (SiNPs) requires toxicity information. Currently, there are knowledge gaps on mechanistic understanding of SiNP toxicity.

Methodology: In this work, we exposed mitochondrial fractions from J774 mouse macrophage cells to well-characterized amorphous pristine (15, 30, 75 nm) and surface-modified (-NH2, -C3COOH, -C11COOH, -PEG) SiNPs (15 nm) at 5 and 15 µg/mL doses (37oC, 2h). Nanoparticle exposed mitochondrial fractions were lysed, clarified by molecular weight fractionation, enzymatically digested and tryptic peptides were analyzed by LC-mass spectrometry (orbitrap) to explore SiNP exposure-related protein changes. Bioinformatics on proteomic data was done using Ingenuity Pathway Analyses. Furthermore, J774 cells were also exposed to these SiNPs, cytotoxicity (e.g. ATP) testing was conducted and TEM analysis was done to assess subcellular localization of SiNPs.

Results: Our results showed SiNP size– and surface modification-related changes in various mitochondrial proteins (n≥200) at the two exposure doses tested. Some of these protein changes were related to respiratory complex proteins (e.g. cytochrome c oxidase, ATP synthase subunit, electron transfer flavoprotein subunit) and oxidative stress (e.g superoxide dismutase), and exposure dose-related changes were also seen in these protein responses. TEM analyses revealed internalization of SiNPs in J774 cells and their localization on subcellular organelles such as mitochondria. Also, particle size and surface modification-related cytotoxicity changes were seen.

Conclusions: Our findings reveal that SiNP size and surface functionality can impact on mitochondrial pathways consistent with decreased cellular ATP levels in J774 cells exposed to SiNPs, demonstrating the use of proteomic analyses in toxicity screening and in obtaining mechanistic information on SiNP toxicity in vitro.

P082

Update on LM-GlycomeAtlas: An integrated visualization for mouse tissue glycome mapping data with lectin histochemistry

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Topic: Cellular and Spatial Proteomics

Introduction: To obtain quantitative and qualitative information of glycan structures expressed in microscopic regions, using laser microdissection-assisted lectin microarray, we have established a glycomic profiling method of formalin-fixed paraffin-embedded tissue sections (1). For effective visualization of this "tissue glycome mapping" data, we have developed a novel online tool called "LM-GlycomeAtlas" (https://glycosmos.gitlab.io/lm-glycomeatlas). In Version 1.0, we have provided one dataset of glycomic profiles obtained from nine tissues of normal mice (2). Here, we introduce the updated versions; Version 1.1 which includes additional deposited data and Version 2.0 which visualizes staining images with multiple lectins on the array.
Methods: We employed two published datasets obtained from five tissues of normal C57BL/6J mice (1) and from cardiac tissues of dilated cardiomyopathy model and age-matched normal mice (3). Versions 1.1 and 2.0 were implemented essentially by modifying Version 1.0 built using HTML5. In Version 2.0, histological images were displayed using OpenSeadragon (http://openseadragon.github.io) and D3 (https://d3js.org).
Results: A total of 209 glycomic profiling data were additionally deposited in Version 1.1. Furthermore, Version 2.0 provides additional histological images of cardiac sections stained independently with nine lectins that showed higher signals ($P < 0.05$) in diseased hearts compared to those of normal hearts (3). These interfaces allow users to display multiple histological images of interest (e.g. diseased and normal mice), facilitating the evaluation of tissue glycomic profiling.
Conclusions: This tool was successfully updated to deposit multiple datasets of glycomic profiling with high-resolution histological images. By integrating with other tools for glycoproteomic data and protein glycosylation machinery, available in the GlyCosmos Portal (https://glycosmos.org) (4), this tool will be one of the most valuable open resources that contribute to the glycoscience community.

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P083

Spatially resolved proteome profiling of FFPE tissues

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Topic: Cellular and Spatial Proteomics

Introduction: Clinical tissue samples are routinely formalin-fixed, and paraffin embedded (FFPE) as a cost-effective way of storing tissue biopsies. Hence, these samples represent an invaluable source of specimens for clinical proteomics research. However, extracting proteins from these samples for analysis by mass spectrometry has been challenging especially for spatially resolved proteomic studies due to extensive crosslinking. We have previously developed nanoPOTS (nanodroplet processing in one pot for trace samples) an effective platform for nanoscale and single cell proteomics. Here we present a nanoPOTS-based workflow for FFPE tissue samples that provides a high degree of proteome coverage for spatially resolved protein quantification.

Method: FFPE samples of human prostate xenograft tissue (10µm thick) were laser micro dissected to 200µm squares. Using these samples, we evaluated the impact of extraction solvent (trifluoroethanol, n-Dodecyl-β-D-maltoside (DDM)), temperature (65-95C) and time (1-3hrs) to maximize proteome coverage. Results: With our optimized FFPE nanoPOTS sample preparation protocol, using extraction in 0.1%DDM for 3hrs at 70C, we identified an average of 8000 peptides and 1500 protein groups from the 200µm tissue samples using MaxQuant with a 1%FDR. This exceeds the coverage achieved previously for similarly sized samples.

Conclusion: This study demonstrates the feasibility of achieving spatially resolved proteomics for FFPE samples which constitutes the large majority of preserved clinical tissue biopsy. These proteomic analyses, which has previously been constrained by the availability of much less common fresh frozen tissue biopsy samples, can now conceivably be made available for a far broader spectrum of clinical investigations in the biomedical field. We will also be applying this condition to a fully automated sample preparation and LC/MS system and show applicability by performing comparative study using clinical biological samples.

P084

Combining SDS with Subcritical water extraction for improved recovery of intracellular proteins

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Topic: Cellular and Spatial Proteomics

Introduction: Subcritical water (SW) is defined as water at temperatures above 100 oC but below the critical point (374°C) and requires sufficient pressure to maintain a liquid state. Previous studies have shown the potential of SW for high yield protein extraction, though thermal denaturation or degradation becomes a concern following extended incubation at high temperatures. Sodium dodecyl sulfate (SDS) is a detergent favored for protein solubilization. Here we examine the combination of SDS with SW extraction as a means of maintaining protein solubility at high temperature while preventing thermal degradation through rapid extraction.

Method: S. cerevisiae and Schizachyrium spp. were subject to extraction inside a sealed stainless steel tube at various temperatures up to (180°C) with incubation ranging from 10 to 30 minutes, with or without inclusion of SDS (up to 2%). Protein recovery was determined by BCA assay, with SDS PAGE used to monitor protein degradation. Bottom-up proteome analysis with LC-MS/MS will be utilized to classify the extracted proteins and further assess the integrity of extracted protein.

Results: Extraction efficiency of yeast proteins in SW is significantly enhanced with the addition of SDS up to 2%. The optimal temperature for SW extraction was 150oC, with a calculated protein recovery up to 80%. SDS PAGE analysis revealed distinct protein bands over a range of molecular weights, suggesting that intact proteins are preserved through the SW extraction process. Further assessment of protein extraction and thermal stability will be conducted by mass spectrometry. As well, the SW + SDS extraction protocol will be applied to isolate proteins from algae, which represents a more challenging samples containing a more rigid cell wall.

Conclusion: The combination of SDS with subcritical water enhances the extraction efficiency of proteins in yeast. Further testing will be used on yeast to determine if proteins thermally degraded.

P085

Proteomics of Human Milk: Definition of a Discovery Workflow for Clinical Research Studies

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Topic: Food and Nutrition

Introduction: Milk is a complex biological fluid composed mainly of water, carbohydrates, lipids, proteins, and diverse bioactive factors (1). Human milk represents a unique tailored source of nutrients that adapts during lactation to the specific needs of the developing infant. Proteins in milk have been studied for decades and proteomics, peptidomics, and glycoproteomics are the main approaches previously deployed to decipher the proteome of human milk at large. Based on our experience in analyzing biological fluids with liquid chromatography-mass spectrometry (LC-MS), we aimed at implementing a highly automated proteomic pipeline for analysis of human milk in clinical research.

Methods: Commercial human milk samples were used to optimize workflows. Centrifugation defatted milk samples and was assessed before and after reduction, alkylation, and enzymatic digestion of proteins, with and without presence of surfactants. Skimmed milk samples were analyzed by label-free (LF) and isobaric labeling-based (ILB) quantitative (Q) MS on an Orbitrap Fusion Lumos. Sample fractionation using isoelectric focusing was evaluated. The most appropriate workflow was transferred to a liquid handling workstation for automated sample preparation (2).

Results: While LFQ approaches provided largest proteome coverages, coverage consistencies were higher using ILBQ. The introduction of a surfactant before defatting significantly improved milk proteome coverage. Using fractionation, we identified more than 2000 proteins in human milk. Focusing on ILBQ using tandem mass tags, we demonstrated reproducibility of quantitative measurements. Averaged coefficients of variation for proteins were 10%, 11% and 16% at LC-MS injection, ILBQ experimental batch and individual sample levels, respectively, when using a liquid handler.

Conclusion: We have defined a highly automated proteomic workflow for human milk analysis that we will now deploy in clinical research.

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P086

Proteomics and metabolomics analysis of wine from the Hungarian "Tokaj" wine region

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Topic: Food and Nutrition

Introduction:
Wine is a complex medium containing alcohol, different types of acids, and macromolecules including polysaccharides and proteins. The protein, even it is found in low concentration, is one of the most important components of the wine which makes a significant contribution to the wine quality, stability, and taste. Protein and free amino acids can be originated from grape and yeasts. For decades, protein and metabolite analysis of the wine has revealed the health benefits of wine components.
In this study, we aimed to evaluate the proteins and the level of 20 protein forming free amino acids of two wine types from the Hungarian "Tokaj" region.

Methods:
We examined two white wine types from the Hungarian "Tokaj" wine region, a UNESCO world heritage site. Wine samples were filtered by the 3kD Nanosep filter and used for the amino acid analysis. Free amino acids were derivatized with the AccQ-Tag derivatization kit (Waters) and the concentrations of amino acids were determined with the H-Class UPLC system (Waters). The raw data were processed with Empower 3.0 software.
For protein analysis, in solution trypsin digestion was performed and the peptides were examined with Orbitrap Tribrid (Thermo Scientific) mass spectrometry system coupled with Easy-nLC UPLC (Thermo Scientific). For data analysis, Max Quant and Scaffold (Proteome Software Inc.) software were used and the results were subjected to network analyses with the help of String 11.0.

Results:
The protein and metabolomics analysis revealed differences between examined wine types.

Conclusion:
The protein and metabolite analyses are useful methods for the examination of protein and free amino acid components of wines.

P087

Glycoproteomics of Saccharomyces cerevisiae yeast cell wall mannoproteins

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Topic: Food and Nutrition

Yeast cell wall (YCW), the outermost organelle of the yeast cell, is composed of an inner polysaccharide layer, to which are bound mannoproteins. These latter forming the YCW outer layer are its second most abundant component (40%, w/w). YCW mannoproteins are heavily mannosylated (50-90%, w/w) by both short simple O-linked glycans and complex N-linked glycans. They have functional and health promoting properties, but have been little investigated. This work aims to study YCW mannoproteins at the molecular level based on mass spectrometry (MS) and capillary electrophoresis (CE) techniques.

SDS-extracted YCW samples obtained by mechanical disruption of yeast samples were O- using NH4OH or Canavalia ensiformis mannosidase and N-deglycosylated by PNGase F/Endo H with an adapted eFASP method. The resulting peptides were analyzed by nanoESI-LC-MS/MS. Proteins were identified using Proteome Discoverer 2.2 against Saccharomyces Genome Database (S288C strain) dataset. Subcellular localization of identified proteins was determined by Gene ontology analysis. O- and N-glycans were chemically derivatized by aminative reduction reaction and analyzed by CE and microESI-LC-MS. Glycosylated peptides were identified using Byonic software.

We showed the reliability of the YCW extraction method for YCW enrichment. The N-deglycosylation step has the advantage to increase the sequence coverage of mannoproteins. While chemical O-deglycosylation with NH4OH has drawbacks on decreasing the number of identified peptides and thus mannoproteins (from 32 to 20) and their amount (from 16% to 11%), due to a degradative effect that has been overcome by the use of mannosidase, allowing the identification of 37 mannoproteins composing 37% of quantified proteins. Mannoproteins O- and N-glycans were isolated simultaneously and efficiently permitting their analysis by MS and CE respectively.

This work describes the first a one-pot glycoproteomic methodology allowing mannoproteins' deglycosylation adapted to an eFASP method applied on extracted YCW. It is currently applied to analyze YCW during different yeast growing phases.

P088

Proteomic analysis of the immune-challenged macrophage myddosome: composition, dynamics, and interactome

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Topic: Immunology and Inflammation

Introduction: Toll-Like Receptors (TLRs) are the first line of defense for the innate immune system. TLRs on macrophages and dendritic cells recognize conserved Pathogen Associated Molecular Patterns (PAMPs) to initiate the expression of pro-inflammatory cytokines and interferons through activating transcription factors. MyD88 is the most common adaptor protein for TLR signaling pathways. Upon stimulation, MyD88 binds to additional proteins to form a large signaling complex known as the myddosome. While nine of the TLRs use myddosomes, each pathway elicits distinct secretomes. Investigation of the formation, composition, and regulation of the myddosome may uncover the cause for the distinct responses.

Methods: Immortalized Mouse Macrophages (IMM) were metabolically labeled via Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) for Mass Spectrometry (MS)-based quantitative proteomic measurement of the myddosome during a two-hour time course following stimulation with either LPS, Pam3CK4, R848, or Poly(I:C) that stimulate the TLR4, TLR1/2, TLR7/8, or TLR3 pathways, respectively. Immunoprecipitation of MyD88 and the associated protein complex(es) was performed, followed by tryptic cleavage and analysis with LC-MS/MS on an Orbitrap Velos Pro. protein identification and quantification was achieved through MaxQuant.

Results: Analysis confirmed the IP success and presence of expected associated-proteins in the three MyD88-dependent pathways, such as the IRAK proteins, as well as interactors in ubiquitination and phosphorylation mechanisms such as RPS27A and PRK. The data highlights different rates due to the slower progressions of some protein-protein interactions compared to others. Differing complex rates could delay downstream signal transduction.

Conclusions: Differing complex dynamics suggest additional regulatory signaling elements help account for changes in secretome responses between TLR pathways. We hypothesize these differences could be due to PTM variations between the responses and are currently examining the PTMs that occur following stimulation.

This work was supported in part by the Intramural Research Program of the NIH, NIAID.

P089

A time resolved Secretome Analysis of Hepatocytes during Acute-Phase Response

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Topic: Immunology and Inflammation

Introduction: Hepatocytes are the major parenchymal cells in the liver and play essential roles in metabolism, detoxification, protein synthesis and innate immunity. In steady state, hepatocytes constitutively express and release a large variety of proteins into the blood stream that perform pivotal functions in innate immunity and homeostasis. Upon perturbations, such as inflammatory processes, hepatocytes receive inflammatory signals that initialize the acute-phase response (APR) and stimulate the secretion of proteins with immunomodulatory functions.

Methods: We employed a scalable high-throughput and sensitive TMT-based proteomics workflow to precisely monitor quantitative and time-dependent changes of protein secretion upon activation of the APR. Our label- and serum-free protocols minimize perturbations by metabolic labelling reagents, but serum-free culturing limits the observable time window and can interfere with cell viability and signaling. We employed a modified experimental design where treatments were performed in presence of serum, and secretion was probed in a two hours serum-free collection window, which enabled secretome studies over several days.

Results: We tracked the time-dependent secretion of proteins in the liver cell lines HepG2- and HepaRG upon stimulation of the APR with the pro-inflammatory cytokines IL1β, IL6 and TNFα. The secretion pattern from differentiated HepaRG cells contained the highest number of bona fide APR proteins. Distinct time- and stimulus-dependencies could be observed. IL1β stimulated HepaRG-cells showed greater diversity in the secretion of APR- and inflammation-related proteins compared to the cytokines IL6 and TNFα. IL1β- and TNFα-treatments resulted in the secretion of C-reactive protein (CRP) from HepaRG-cells but not from HepG2-cells and simultaneously caused secretion of the APR key mediator IL6.

Conclusions: We provide a quantitative and dynamic catalogue of proteins that are differentially released by hepatic cell lines during APR. Our results highlight the complexity of the inflammatory secretome and give a systems biology perspective of hepatocytes during inflammatory processes.

P090

B cell and T cell proteomics for monitoring long term immune system dynamics

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Topic: Immunology and Inflammation

Introduction: Many proteomics experiments sample only a single time point, limiting our understanding of individual variation in health and disease. With this in mind and in preparation for a longitudinal study of disease progression, we characterized variation in healthy lymphocytes over time by comparing B cells to T cells of healthy subjects with monthly collection times.

Methods: Whole blood samples were collected monthly by venous blood draws. Using antibody guided flow cytometry cell sorting, we separated the lymphocytes into 145 cell samples of B cells and samples of T cells. Five replicates of each type were prepared for mass spectrometry for proteomics using the AutoPOTS workflow. Quality checks and comparative analyses were performed to identify variation within and between cell types, subjects, and time points.

Results: On average 1300 proteins were quantified in each sample, comprising proteins involved in both cell type specific immune functions and general metabolic capabilities. Gene set enrichment analysis confirms that these proteins are strongly representative of lymphocytes. Primary component analysis reveals that distinctions between cell types are most prominent, with replicates of a cell type from the same subject having greater similarity.

Conclusions: Small sample proteomics successfully characterize distinctions between lymphocyte subpopulations and different human subjects. Our measurements allow us to characterize general stability over time in lymphocytes from healthy individuals. These data provide context for future work in examining individual specific responses to disease progression.

P091

The DNA Sensor cGAS is Decorated by Acetylation and Phosphorylation Modifications in the Context of Immune Signaling

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Topic: Immunology and Inflammation

Introduction: Cyclic GMP-AMP synthase (cGAS) is a pattern-recognition receptor of the mammalian innate immune system that is recognized as a main cytosolic sensor of pathogenic DNA. cGAS DNA binding initiates catalytic production of cyclic GMP-AMP, which activates the STING-TBK1-IRF3 signaling axis to induce cytokine expression. Post-translational modification (PTM) has started to be recognized as a critical component of cGAS regulation, yet the extent of these modifications remains unclear. Here, we report the identification and functional analysis of cGAS phosphorylations and acetylations in several cell types under basal and immune-stimulated conditions.

Methods: cGAS was enriched by immunoaffinity purification from human primary fibroblasts prior to and after infection with herpes simplex virus type 1 (HSV-1), as well as from immune-stimulated STING-HEK293T cells. PTMs were validated and quantified by parallel reaction monitoring (PRM) mass spectrometry in fibroblasts, HEK293T cells, and THP-1 macrophage-like cells. To assess the functional relevance of each PTM, we generated a series of single-point cGAS mutations that prevented phosphorylation and acetylation or that mimicked the modification state. cGAS-dependent apoptotic and immune signaling activities were then assessed for the mutations.

Results: Six phosphorylations and eight acetylations were detected, of which eight PTMs were not previously documented. The acetyl-mimic mutations at Lys384 and Lys414 inhibit the ability of cGAS to induce apoptosis. In contrast, the Lys198 acetyl-mimic mutation increased cGAS-dependent interferon signaling when compared with the unmodified charge-mimic. Moreover, targeted PRM quantification showed that Lys198 acetylation is decreased upon infections with two herpesviruses—HSV-1 and human cytomegalovirus (HCMV). By determining the protein interactomes of the cGAS mutants following HSV-1 infection, several associations were identified that can contribute to the functional difference of Lys198 acetylated cGAS.

Conclusion: cGAS is decorated by numerous functional acetylations and phosphorylations, and previously unrecognized Lys198 acetylation provides a regulatory point for modulating cGAS-mediated immune signaling during viral infection.

P092

Proteomic analysis of human corneal epithelial cells infected with Aspergillus flavus spores

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Topic: Infectious Diseases

Introduction:
Corneal ulcer due to trauma or infectious keratitis is the leading cause of monocular blindness affecting two million people worldwide and is endemic in tropical regions. Aspergillus flavus next only Fusarium species is one of the predominant causative agents for fungal keratitis. This study explores the proteomics analysis of fungal cell wall component beta glucan (Zymosan) and A.flavus spores exposed to human corneal epithelial (HCE) cells.
Methods:
HCE cells ($1x10^6$) were treated with zymosan (100 & 200 µg/ml) for 12hrs (Guo et al., 2009; Fekkar et al., 2012) and the isolated total cell proteins were analyzed by 2D-DIGE. The cells were infected with A.flavus spores at 1:10 MOI and examined the formation of F-actin ring by TRITC-phalloidin staining. Maturation of conidia was monitored using antibodies specific to endosomal markers by immunofluorescence analysis. Mass spectrometry was carried out to identify infection induced proteins and proteome of phagosome containing conidia.
Results:
The 2D-DIGE analysis showed that zymosan didn't induce changes in the proteome of HCE cells and might be due to the sensitivity of DIGE. While the A.flavus spores bound to HCE cells, the HCE cells formed a ring like structure around the A.flavus conidia by F-actin. Further the conidia in the phagosome matured by acquiring early (CD71) and late (LAMP1) endosomal markers. Mass spectrometry showed that the infected proteins are majorly involved in the UPR signaling and targeted proteomics revealed the identification of endosomal proteins to the phagosomes containing conidia.
Conclusion:
HCE cells internalizes A.flavus spores as good as professional phagocytic cells and might participate in antifungal innate immunity. However, under our experimental conditions, HCE cells failed to response to zymosan.
References:
1. Guo H., et al. 2009. FEMS Immunol Med Microbiol 56:88-93
2. Fekkar A., et al. 2012. J. Infect. Dis 205:1163-1172

P093

SARS-CoV-2 Spike Interactome Promiscuously Facilitates Epithelial Cell Infection

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Topic: Infectious Diseases

The high infectivity and transmissibility of the coronavirus SARS-CoV-2 is responsible for the current COVID-19 pandemic, which challenges human, social, and economic health worldwide. In search of drugs that curb COVID-19, we identified the interactomes of the virus receptor Spike protein, and additional 2xStrep-tagged SARS-CoV-2-ORFs (1) in disease-relevant human bronchial epithelial cells (HBE16o–). The Spike interactome was purified according to CoPIT (2), digested with trypsin, and the resulting peptides separated by reversed phase HPLC (Evosep). Peptide identification with a timsTOFpro mass spectrometer (Bruker) afforded >200 interactors of the SARS-CoV-2 proteins analyzed. We show that tight junction proteins are part of the Spike interactome and may support virus cell entry and exit. However, these proteins are unaltered in the interactome of the Spike variant D614G which is prevalent in the US and Europe and exhibits increased infectivity (3). We found that G614 Spike recruits more interactors that facilitate its translation at the rough endoplasmatic reticulum (ER) and binds to fewer proteins of the ER quality control than the original D614 variant. Our results might explain why G614 yields 10-fold more Spike protein per mature virus particle than D614 (4). Because the Spike protein interactome determines which cell types SARS-CoV-2 can infect, or the virus tropism, we devised a novel, proteome based cell type set enrichment analysis (pCtSEA) which indicated that ciliated epithelial cells in the lung are the most susceptible to SARS-CoV-2 infection. Furthermore, pCtSEA revealed that a subset of macrophages as well as endothelial and epithelial cells like keratinocytes might be vulnerable to SARS-CoV-2. In summary, the Spike interactome elucidated molecular mechanisms that guide SARS-CoV-2 infectivity and revealed a broad virus tropism which may explain a slow recovery and long lasting side effects from COVID-19 in some patients.

(1) Nature doi:10.1038/s41586-020-2286-9
(2) Nature doi:10.1038/nature15729
(3) Cell doi:10.1016/j.cell.2020.06.043
(4) bioRxiv doi:2020.2006.2012.148726

P094

623 plasma samples defined blood-based signature for COVID-19 positivity and progression

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Topic: Infectious Diseases

Introduction: We designed a two-part study to (i) establish reference ranges of plasma proteins (ii) differential protein expression study in Covid-19 positive individuals who were compared to disease controls (negative individuals presenting Covid-19 symptoms) and healthy controls using highly optimized automated system for maximized reproducibility.

Methods: 623 native plasma samples were processed using i7 automation workstation protocol (Beckman-Coulter) and analyzed using data independent discovery workflow on Evosep coupled to Exploris 480 system (Thermo Fisher) using a 21 min gradient time. N=100 samples were healthy (HC), n=191 were disease control (Neg), n=191 individuals were Covid-19 positive (Pos). 141 Pos individuals had their blood collected at the three sequential timepoints (t1, t2 and t3). Data was processed with OpenSwath [1] and mapDIA software [2]. Further statistical analyses were run using an R package. Batch and individual sample QC were evaluated by QuiC (Biognosis) and in-house built Sample-Processing and MS-Summary software.

Results: For maximum assay performance within and across batches, a pooled plasma sample was monitored to ensure data quality within a CV threshold of 20%. Cluster analysis revealed protein signatures (4 clusters of 52 proteins) that clearly separated individuals diagnosed as Covid-19 positive from healthy controls and to a lesser degree from the disease controls. It was also possible to identify a panel of proteins whose temporal expression changed within the blood of individuals collected at t1 versus t3 (79 proteins, <0.01 FDR) including immunoglobin seroconversion. Pathway enrichment analysis revealed that those proteins driving the discriminatory effect were involved in coagulation, complement activation, dyslipidemia and the humoral response.

Conclusion: Reproducible, high-throughput DIA proteomics is achievable. Large sample number facilitated the identification of potentially biologically relevant proteins in plasma from individuals with COVID-19 versus their disease counterpart. Proteins identified could discriminate between healthy and diseased states as well as differences in disease progression.

P095

Influence of antibiotic therapy on lung proteome adaptation in murine pneumonia

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Topic: Infectious Diseases

1. Introduction

Community-acquired pneumonia (CAP) remains a major cause of death worldwide, with Streptococcus pneumoniae as the most common causative pathogen. It is known, that an early antibiotic treatment start is crucial for a positive patient outcome. Nearly 30% of all CAP-associated deaths appear during the first 48h after hospital admission. Therefore, we analysed the lung proteome in a murine CAP model with early (24h p.i.) and late (48h p.i.) antibiotic treatment. We aimed to uncover specific changes in protein abundance patterns and affected biological pathways associated with the time point of treatment.

2. Methods

Mice were infected with 5x106 colony forming units of S. pneumoniae and received 0.4 mg ampicillin twice daily starting 24h and 48h post infection. We analyzed lung tissue samples of infected and control animals with an optimized SP3 bead-based digestion protocol using a Q Exactive™ Plus mass spectrometer in data-independent acquisition mode.

3. Results

Both, early and late antibiotic intervention successfully reduced bacterial burden. Only early antibiotic therapy prevented lethal disease outcome. During S. pneumoniae infection especially CD177 antigen, neutrophil cytosol factor2, and myeloblastin showed a significant intensity increase. Early antibiotic treatment prevented intense activation of acute phase response and FXR/RXR and LXR/RXR activation and thus altered the inflammation process. Proteins involved in complement system, acute phase response, lipid metabolism, and coagulation revealed profound differences in their abundance in animals treated only 48h after infection compared to naive mice.

4. Conclusion

Data revealed that late or untreated infected mice displayed profound increased levels in proteins involved in barrier functions or immune defence. Only after early antibiotic treatment proteins fulfilling these

functions showed altered abundance, and thus, could contribute to improved survival. Our findings contribute to better understanding molecular adaptation to CAP and suggest investigating membrane stabilising therapeutics to prolong the time of effective antibiotic treatment.

P096

MS-based Sars-Cov-2 protein detection in patients (Cov-MS): a successful community effort

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Topic: Infectious Diseases

Introduction:
Rising population density, global mobility and insufficient health care are among the reasons why pathogens such as SARS-CoV-2 spread so rapidly across the planet. The policy response to such pandemics will always have to include accurate monitoring of this spread, as this provides one of the few alternatives to total lockdown. However, COVID-19 diagnosis is currently performed almost exclusively by RT-PCR. Although this is efficient, automatable and acceptably cheap, it is not justified for the world to rely on only one type of technology. Not only are reagents in short supply, but the test is also difficult to validate. We therefore developed an alternative diagnostic test that detects SARS-CoV-2 proteins using mass spectrometry (MS). As this test relies on viral proteins instead of RNA, it provides an orthogonal approach to RT-PCR, using other reagents that are cheap and widely available, as well as currently idle personnel and instruments.

Methods and results: An initial discovery phase of two weeks on high-resolution Q-TOF instruments was translated into a sensitive 8-minute targeted MRM-assay on clinically applicable tandem quadrupole instruments. This assay resulted in 100% accuracy on 20 blind-coded clinical samples with a very strong correlation between the MS signal intensity and the Ct-value from RT-PCR. To increase applicability, sensitivity and robustness, we established the Cov-MS consortium, consisting of more than 10 different European labs and several industrial partners. By providing them with a Standard Operating Procedure (SOP) for transition selection, an open source software template containing the 17 target peptides (Skyline) and a sample kit containing recombinant viral proteins, they could efficiently select the best targets for their lab and MS instrument.

Conclusion: Cov-MS was validated on instruments from four major MS-vendors. With minimal testing capacity of 150 samples per day per instrument, our assay can contribute significantly to future diagnostic capabilities.

P097

Proteome analysis of Streptococcus suis under stress conditions and in host-pathogen interaction

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Topic: Infectious Diseases

Introduction. Streptococcus suis, as a commensal of pigs, can cause invasive infections and is responsible for high economic losses in swine farming worldwide. As an important zoonotic pathogen S. suis is able to induce meningitis and septicemia. The species comprises 29 serotypes of which serotype 2 is the most prevalent serotype, followed by serotypes 9, 7 and 3 in Europe [1]. The study is focused on a comparative profiling of S. suis proteome patterns and immunoproteome screening of S. suis antigens to reveal the adaptation to host niches and to understand the switch from commensal to an invasive pathogen.

Methods. Proteome analysis of serotype 2, 9 and 7 was carried out with a data-independent acquisition (DIA) mass spectrometry workflow. Moreover, S. suis specific antibody profiles of potentially immunogenic proteins were investigated in a multiplexed suspension bead array.

Results. For proteome analysis a spectral library was generated using strain-specific genome sequences and S. suis protein extracts from samples grown in different media, at different temperatures and after nutrient or iron limitation. Optimized peptide preparation from low bacteria numbers (10^6 – 10^7) [2] from ex vivo samples (pig CSF and plasma) and after recovery from infection allowed monitoring of around 1100 proteins using the DIA-workflow. The proteome profiling revealed differentially abundant proteins of different cellular pathways. In addition, the antibody response of infected pigs against S. suis antigens was quantified by bead-based immunoproteomics. The study revealed proteins, which might be involved in adaptation to the different host niches and constitute potential new virulence factors and vaccine candidates.

Conclusion. A combination of immunogenic antigens of different strains specific for the host compartments (CSF and blood plasma) will be selected and screened for suitability as a multicomponent vaccine.

[1] Segura M et al., Virulence 2017
[2] Blankenburg S et al., Proteomics 2019

P098

CD8+ and CD4+ T Cell responses of COVID-19 patients are shaped by the SARS-CoV-2 epitope-associated mutational landscape

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Topic: Infectious Diseases

Introduction: Understanding the impact of SARS-CoV-2's global mutational landscape on epitope presentation and CD8/CD4 T lymphocyte-based immunity is of great importance for vaccine development in the context of a rapidly spreading pandemic.

Methods: We identified missense SARS-CoV-2 mutations by aligning over 68,000 SARS-CoV-2 genomic sequences across 143 countries (GISAID) to the reference sequence, Wuhan-1 (NC_045512). We then used netMHCpan 4.0 (HLA class I) and netMHCIIpan 4.0 (HLA class II) to compare the predicted binding of the mutated and unmutated forms of all possible mutation-containing peptides presented by common class I and II HLAs. Finally, we are in the process of validating the predicted impacts of mutations on peptide presentation and adaptive immune responses through MHC binding assays as well as ELISpots (using COVID +/- PBCMs).

Results: We identified 2807 missense SARS-CoV-2 mutations occurring in at least 5 individuals. Among those mutations, 49 were observed in >1% SARS-CoV-2 sequences in GISAID. Strikingly, our findings show that the SARS-CoV-2 mutational landscape is not randomly generated but rather governed by defined mutational patterns. Specifically, we observed that three residues (Alanine, Proline, Threonine) were preferentially removed, whereas two residues (Phenylalanine, Isoleucine) were preferentially introduced. Using this information along with known binding motifs, we identified a variety of prevalent SARS-CoV-2 mutations predicted to lead to the loss of epitope-HLA binding. These include 11083G>T, 25350C>T, 25563G>T, and 23403A>G, leading to the loss of TQWSLFFFL (A*02:01), SEPVLKGVKL (B*07:02), FQSASKIITL (A*02:06), and SNQVAVLYQDVNCTE (DRB4*01:01) respectively.

Conclusion: Our data provide ground-breaking information regarding SARS-CoV-2 mutational patterns, and the implication of numerous frequent SARS-CoV-2 mutations in the loss of class I/II epitopes. Upon validation with T cell assays, this work will impact vaccine design and provide information regarding HLA-specific susceptibility to mutated forms of SARS-CoV-2.

P099

SignalScan™ targeted LCMS/MS assay to profile changes in protein and phosphorylation levels with SARS-CoV-2 infection and treatment

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Topic: Infectious Diseases

Infection with the novel coronavirus SARS-CoV-2 has resulted in a worldwide viral pandemic. Here we present a targeted LCMS/MS-based assay to measure changes in cell signaling with viral infection and treatment. Assay targets represent viral and host proteins, including the SARS-CoV-2 Spike and Nucleocapsid proteins and human proteins such as ACE2, cytokines, and intracellular signaling markers, which are involved in viral entry, the innate immune response, interferon signaling, translational activity, and viral RNA detection. Changes in total protein levels, sites of phosphorylation, and cleavage events are quantified.

We synthesized and quantified a mixture of over two dozen heavy labeled (13C, 15N) Stable Isotope Standard (SIS) peptides and phosphopeptides representing both viral and host proteins. In the SignalScan workflow, one pmol of each SIS peptide was spiked into one microgram of tryptic peptides derived from a human or primate cell line, LCMS/MS was performed on a Fusion Lumos mass spectrometer using the SureQuant SIS-Assisted Scanning (SISAS) method, and data analysis was performed in Skyline.

Cells were mock-infected or infected with SARS-CoV-2 and treated or untreated with a kinase inhibitor drug candidate prior to lysis and digestion. Quantitative profiling of viral peptides and phosphopeptides revealed decreases in viral protein phosphorylation upon kinase inhibitor treatment without impact to viral protein levels. Host protein expression and phosphorylation changes were also measured. The SISAS method for targeted analysis resulted in improved coverage of assay targets over data-dependent analysis.

Utilizing the SignalScan workflow enables deeper, more consistent profiling of target peptides for accurate quantification of both protein levels and post-translational modifications in unenriched samples, greatly simplifying both sample preparation and reducing starting sample requirements. This simplified protocol together with available files for both MS method setup and Skyline analysis enable researchers to easily assay critical viral and host signaling events.

P100

Translational COVID-19 serology in home-sampled blood

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Topic: Infectious Diseases

Introduction: The COVID-19 pandemic has posed tremendous challenges for the global community and health care. To determine the prevalence of SARS-CoV-2 infections in an unbiased manner and outside of clinical routines, we established a translational serology approach that made use of precision blood sampling at home and multiplexed antibody assays.

Methods: During early and late April, we mailed sets of 1000 kits with blood collection cards (qDBS, Capitainer AB) to random households in Stockholm. Blood samples were obtained by the laypersons via finger-pricking to collect 2x 10 µl per card. The dried blood samples were then returned by regular mail and their eluates were analyzed for IgG and IgM using a bead-based assay with several SARS-CoV-2 proteins and antigens from other viruses. The assay and workflow were evaluated with PCR-positive and pre-pandemic samples, benchmarked against a commercial ELISA, and blood samples collected from venous draw by trained personnel.

Results: After receiving 55% (1097/2000) of the cards back within three weeks, 80% (878/1097) were deemed suitable for serological analyses. Profiles of IgG and IgM were obtained from the same eluates in sets of 180 samples per batch. The obtained data showed noticeable diversity of the immune response against the S, RBD and N proteins. When applying unsupervised dimensionality reduction and combing data from IgG and IgM, we deemed 4% of the samples collected during mid April (19/435; 95% CI: 2.4%-6.3%) and 6% collected early May (28/443; 95% CI: 4.1%-8.6%) as seropositive.

Conclusion: Analyses of home-sampled blood revealed valuable insights about SARS-CoV-2 seroprevalence and the patterns of immune response against its antigens. This showed that combining multiplexed assays with home-sampling of blood can provide a viable strategy towards individual-level assessment of seroprevalence, follow changes in the immune systems, and support the efforts towards effective vaccine.

Reference: Roxhed et al (2020) medRxiv 20143966.

P101

Computational identification of human biological processes and protein sequence motifs putatively targeted by SARS-CoV-2 proteins using protein-protein interaction networks

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Topic: Infectious Diseases

Introduction: Despite the ongoing efforts against the COVID-19 pandemic, knowledge of the interactions of the causative virus, SARS-CoV-2, with human host cells remains limited. Investigating protein-protein interactions (PPIs) between viral and host proteins is crucial to provide a better understanding of the mechanisms of viral infection and potential drug targets. We therefore performed an in-depth computational analysis of the recently published interactome of SARS-CoV-2 proteins with human proteins in infected HEK293 cells [1] to reveal the putative molecular pathways affected by the virus and potential host protein binding sites.

Methods: We supplemented the virus-host PPI network with high-confidence PPIs from the STRING database and then used a novel implementation of our GoNet algorithm [2] to identify significantly clustered Gene Ontology (GO) terms in the network. These GO terms represent potential host processes affected by SARS-CoV-2 proteins. Finally, we developed a novel graph theory-based approach (LESMoN-Pro) based on our tool LESMoN [3] to discover protein sequence motifs that are locally enriched in the network and could represent putative host binding sites of SARS-CoV-2 proteins.

Results: Our approach detected 329 GO terms for which the SARS-CoV-2-interacting human proteins are significantly clustered. Some unique GO terms such as those related to the centrosome, cell division, and transcription factor binding, among others, were identified by GoNet. Moreover, we identified 9 putative host protein binding sites using our new LESMoN-Pro algorithm.

Conclusions: Our analyses provide insights into the biological processes and protein sequence motifs that are putatively impacted in human cells during SARS-CoV-2 infection. These observations will help understand the mechanisms of SARS-CoV-2 cell infection and could lead to potential therapeutic targets.

1.Gordon, D. E. et al. 2020 Nature 583, 459–468
2.Lavallée-Adam, M. et al. 2010 J. Comput. Biol. 17, 443–57
3.Lavallée-Adam, M. et al. 2017 Nucleic Acids Res. 45, 10415–10427

P102

The antiviral sirtuin 3 bridges protein acetylation to mitochondrial integrity and metabolism during viral infection

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Topic: Infectious Diseases

Introduction: Regulation of mitochondrial structure and function is a central component of numerous viral infections as a virus means to modulate cellular metabolism and immune responses. During infection with human cytomegalovirus (HCMV), mitochondria undergo fragmentation and alterations in composition. Accumulating evidences have placed mitochondrial protein acetylation into the spotlight, given the HCMV-induced global elevation of the mitochondrial acetylome and the antiviral function of the mitochondrial deacetylase SIRT3.

Methods: To determine the temporal dynamics of SIRT3-substrate interactions, we performed immunoaffinity-purification coupled to mass spectrometry (IP-MS) throughout the course of HCMV infection. The interactions were subsequently overlaid with temporal mitochondrial proteome and acetylome information to identify proteins of potential interest. The impact of SIRT3 and its substrates on mitochondrial integrity and viral progression were investigated using mutagenesis, siRNA-mediated knockdowns, microscopy, and virology assays.

Results: We first establish that SIRT3 deacetylase activity is necessary for suppressing virus production, and that SIRT3 maintains mitochondrial pH and membrane potential during infection. Furthermore, modulation of SIRT3 protein levels or enzymatic activity is sufficient for regulating mitochondrial filamentous structure. By generating temporal interaction datasets, we find altered SIRT3 associations with the mitochondrial fusion factor OPA1 and acetyl-CoA acyltransferase 2 (ACAA2), concomitant with changed acetylation levels. Through microscopy analysis, we discover that OPA1 modulates mitochondrial morphology of infected cells and inhibits HCMV production, which is dependent on its K931 acetylation state, a site regulated by SIRT3. Moreover, we uncover a virus restriction function for ACAA2, an enzyme regulating fatty acid beta-oxidation.

Conclusions: We highlight SIRT3 as a regulatory hub for mitochondrial acetylation, integrity, and metabolism during HCMV infection, and point to global acetylation as a reflection of mitochondrial health. Integrating SIRT3-substrates interactions, mitochondrial acetylome, and proteome facilitates the discovery of factors mediating SIRT3 antiviral function.

P103

Profiling pathogenicity mechanisms of Bordetella pertussis & B. parapertussis, the causative agents of whooping cough, a re-emerging disease, by proteomics

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Topic: Infectious Diseases

Introduction: Even in fully vaccinating countries, whooping cough caused by Bordetella pertussis (Bp) and B. parapertussis (Bpp), is regarded as re-emerging disease. Besides vaccine inefficiency, intracellular pathogen survival leads to dissemination in the population. In order to elucidate bacterial adaptation to infection related conditions, we have analysed the global proteome of both pathogens to iron-limitation and upon internalization of Bp.

Methods: Bp Tohama I, an isogenic Δhfq mutant and Bpp CN2591 were cultivated in iron-replete and iron-depleted Stainer-Scholte medium until late exponential growth. Bacterial proteomes were analysed by nanoLC-MS/MS. In addition, human monocytic THP-1 cells were infected with Bp Tohama I (MOI 150). After 2 h, non-internalized bacteria were killed with polymyxin B. The proteome of recovered intracellular Bp was examined 3 h and 48 h post infection and compared to extracellular Bp grown under similar conditions. Additionally, infection assays were repeated with mutants in BP0414 or hfq, encoding an MgtC homologue or the RNA-chaperone Hfq to assess their impact onto intracellular survival by bacterial counting.

Results: Hfq modulates 33% of Bp proteins regulated during iron limitation (Alvarez Hayes et al., J Proteomics, 2020) and influences bacterial survival. Upon internalization, 40% of the detected proteins showed altered levels including proteins involved in stress response, iron uptake, metabolism, and virulence (Lamberti et al., J Proteomics, 2016). A follow-up study revealed that MgtC plays a role in the adaptation of Bp to the acidic conditions inside phagosomes (Cafiero et al., PLoS One, 2018). Moreover, we identified proteins of stress resistance and virulence exclusively in Bpp that might help explaining differences in pathogenesis of both species (Oviedo et al., J Proteomics, 2019).

Conclusions: Here, we present comprehensive proteome data of the pertussis causing agents that help to better understand their pathogenicity mechanisms and pave the way for follow-up studies of the infected host.

P104

Computational proteomics of SARS-CoV2 in a One-Health perspective.

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Topic: Infectious Diseases

Introduction.
The end of 2019 marked the beginning of a new pandemic, supported by a novel virus (SARS-CoV-2) that rapidly affected the social and health balance all-over the world. Several molecular investigations are being performed to clarify the viral origin and delineating the cellular and host tropism of the viral agent. However, the investigation of the sole genetic sequences may not provide an exhaustive representation of the viral origin, biology and the pathogenetic mechanism.

Methods.
Following the One-Health approach, we investigated the amino acid sequence analogies existing between the main structural proteins of SARS-CoV-2 and the coronaviruses with tropism for the main sinanthropic/companion animals humans are most frequently interacting with. The studies are based on a computational proteomics approach, enabling the immunological featuring of SARS-CoV2 in a safe, rapid, reproducible and reliable context.

Results.
Linear and conformational epitopes are shared between the current SARS-CoV-2 and the coronaviruses with tropism for the main sinanthropic and companion animals, suggesting a role of the animals in eliciting a partial immunizing protection in subjects previously exposed to animal coronavirus. Epitope sharing detected is also of interest for the optimization of effective diagnostic/screening strategies [1–4].

Conclusion.
Employing a One-Health approach is of paramount importance for developing effective control measures against COVID 19. Computational proteomics enables fast and safe survey of the novel pathogen; nevertheless, empirical validations are desirable to confirm findings obtained by these preliminary studies.

P105

SARS-CoV-2 proteome microarray for mapping COVID-19 antibody interactions at amino acid resolution

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Topic: Infectious Diseases

Introduction: Comprehensive profiling of humoral antibody response to SARS-CoV-2 proteins is essential in understanding host immunity and developing diagnostic tests and vaccines.

Methods: To address this concern, we developed a SARS-CoV-2 proteome peptide microarray to analyze antibody interactions at the amino acid resolution. Serum screening using the SARS-CoV-2 proteome microarray can be performed in 1.5 hours while keeping a good dynamic range (~2 orders of magnitude) and lowest limit of detection (94 pg/mL). The intra- and inter- array R correlations were 0.9992 and 0.9978, respectively, demonstrating that the SARS-CoV-2 proteome microarray has high reproducibility.

Results: With the array, we demonstrate the feasibility of employing SARS-CoV-1 antibodies to detect the SARS-CoV-2 nucleocapsid phosphoprotein. The first landscape of B-cell epitopes for SARS-CoV-2 IgM and IgG antibodies in the serum of ten COVID-19 patients with early infection is also constructed. Using array data and structural analysis, a peptide epitope for neutralizing antibodies within the SARS-CoV-2 spike receptor-binding domain's interaction interface with the angiotensin-converting enzyme 2 (ACE2) receptor was predicted.

Conclusions: All the results demonstrate the utility of our microarray as a platform to determine the changes of antibody responses in COVID-19 patients and animal models as well as to identify potential targets for diagnosis and treatment.

P106

Urinary Exosomes protein profile discriminates different clinical subgroups of children with Idiopathic Nephrotic Syndrome

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Topic: Liver and Kidney Diseases

Introduction: Idiopathic nephrotic syndrome (INS) is the most frequent primary glomerular disease in children, whose main clinical feature is the massive proteinuria, due to an altered renal filtration process within kidney glomeruli. The mainstay treatment for patients are corticosteroids (CS), to which some children respond (steroid-sensitive), others do not (steroid-resistance) and many others become dependent (steroid-dependence). This heterogeneity, together with a poor knowledge of the underlying disease mechanisms, makes the syndrome difficult to be interpreted. In this context, the analysis of urinary exosomes (UE) can be a valuable source of information, since their molecular composition mirrors the pathophysiologic status of the origin cells, including the glomerular ones. Therefore, the aim of this study is to verify the feasibility to use UE of patients affected by INS as a source of predictive indicators of therapy response and/or to clarify the disease etiopathogenesis, through a proteomic approach.

Methods: Exosomes were isolated from the urine of paediatric patients in remission, after drug therapy, then UE protein profiles were investigated both by monodimensional electrophoresis followed by cluster analysis and by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: The protein content of patient UE showed peculiar electrophoresis profiles according to the therapy response. A hierarchical clustering analysis led to build a statistical model able to interpret more objectively each patient UE protein profile, assisting the classification of the patient response to therapy and informing about its possible variations. Moreover, the investigation through LC-MS/MS analysis supported these observations, identifying differential proteins characteristic of each patients group and shedding light on pathological mechanisms involved in the response to CS therapy.

Conclusions: This proteomic approach applied on UE shows to be promising in the study of INS, either to elucidate many aspects of the disease mechanisms and as a support tool for clinicians to interpret the patient CS responsiveness.

P107

Monitoring liver regeneration in individuals from living donor transplantation cases. A serum proteomics study.

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Topic: Liver and Kidney Diseases

Introduction. Living donor liver transplantation (LDLT) started 30 years ago in response to the shortage of deceased donor liver grafts. Upon partial resection, the liver undergoes a complex process that involves a finely tuned network of molecular events and interaction of different cell types to ensure efficient proliferation until restoration of liver size and function (1). Despite the many advantages of LDTD, occasional donor morbidity and mortality still remains an issue. In this study we have analyzed the serum proteome temporal dynamics of seven healthy donors of LDTD to define a molecular map of liver regeneration.

Methods. Serum samples were stored at -80°C. Serum proteins were digested with trypsin (1:20), the resulting peptides were purified by C18 columns and dried in a speed-vac. Upon resuspension the PQ500 Reference Peptide Mix (Biognosys) was spiked-in and SureQuant analysis (2) was performed in an EASYnLC 1200 coupled to an Orbitrap Exploris 480 MS. Data analysis was performed using Skyline-daily and Proteome Discoverer.

Results. Forty-eight serum samples from LDLT donors at different time points after surgery were included in the analysis. In all cases above 500 peptides were identified with a median CV=3.8% observed in pooled replicate controls. Overall, despite the heterogeneity among individuals, significant regulation of serum proteome after partial hepatectomy occurred during the first 72 h, which parallels the timing of the initiation and proliferative phases of human liver regeneration (3). Restoration of proteome profile is observed 8 months after PH.

Conclusions. SureQuant targeted analysis using PQ500 reference standards is an efficient and precise method to study serum proteome. The application of this approach allow mapping the regulation of circulating proteins across liver regeneration phases and recapitulates the molecular and cellular events taking place during this well-orchestrated process. We believe that this knowledge may prove its value to improve LDTD safety.

P108

Critical Assessment of Metaproteome Investigation (CAMPI): a Multi-Lab Comparison of Established Workflows

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Topic: Microbial Proteomics and Metaproteomics

Metaproteomics, the study of the collective proteome within a microbial ecosystem, has substantially grown over the past few years. This growth comes from the increased awareness that metaproteomics can powerfully supplement metagenomics and metatranscriptomics analyses. Although metaproteomics is more challenging than single-species proteomics, its added value has already been demonstrated in various ecosystems such as the human gut microbiome. Various experimental and bioinformatics workflows already exist for dealing with complex protein mixtures from microbial communities and the resulting large mass spectrometry data sets.

Here, we present the preliminary results of the first community-driven, multi-lab comparison of well-established methodologies in metaproteomics: the critical assessment of metaproteome investigation (CAMPI) study. We evaluated the influence of a variety of methodological steps, from sample preparation over mass spectrometry acquisition to bioinformatic analysis. This benchmarking study relied on a simplified human intestinal sample and a complex human fecal sample.

Our preliminary findings demonstrate that independent of the applied methods, the main biological meaning can be inferred from the metaproteome data because the taxonomic and functional annotations were very similar across all samples. This outcome is consistent regardless of whether these taxa and functions were assigned based on protein groups or on peptides, and were robust against differences at the spectrum and peptide identification levels. The performance differences that were observed originate from

different wet-lab methods rather than from different bioinformatic pipelines. The finalized CAMPI study will lay a solid foundation for method benchmarking in metaproteomics and will serve as a valuable key reference for future studies.

P109

"Original" Venomics Spatial Mapping of Venom Toxins by Mass Spectrometry Imaging

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Topic: MS-Imaging

Introduction. Toxic secretions in venomous animals have evolved as highly specialized biochemical weapons in venom delivery systems that are individually composed of numerous proteins and peptides to facilitate predation or defense. Despite extensive research, we have a poor understanding of the functional biology of most animal toxins, including venom production, storage, or delivery as well as morphological features within sophisticated venom-producing tissue. Non-targeted mass spectrometry imaging (MSI) can help to fill the gap of functional venomics, in combination with standard omics' approaches.

Methods. Detailed topographical and histological cross sections of the Egyptian cobra (Naja haje) venom gland, one of the most medically important snakes in North Africa, were prepared to evaluate the localization of numerous venom peptides into both a functional and morphological context. Moreover, we applied combination of venom gland transcriptomics and global proteomics analyses to generate a holistic venom overview. Based on the comprehensive venom database, we performed venom toxin visualization directly off tissue sections by MSI-based mass spectrometry.

Results. The global venom analysis by standard integrative venomics enabled us for a quantitative overview of the venom proteome and show advantages and limitations between different omics' technologies. On basis of a holistic venom database, we performed non-targeted MSI to spatially map venom toxins of different families in discrete areas.

Conclusions. Herein, we show spatial heterogeneity of various toxin classes and on top also homogeneity for several proteoforms of the same toxin family. The underlying data clearly prove that toxins are expressed and stored in a spatially separated manner, which help to place the evolution of venoms toxins into a more ecological and functional context.

P110

MALDI guided SpatialOMx on a timsTOF fleX uncover proteomic profiles of breast cancer subpopulations

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Topic: MS-Imaging

Introduction

SpatialOMx provides the unique opportunity to combine regiospecific information of MALDI Imaging with deep proteomic coverage for biomarker discovery and molecular characterization.

Here, we present SpatialOMx for microproteomic characterization of tumor subpopulations in breast cancer (1). In this efficient workflow, unsupervised segmentation of MALDI Imaging data with SCiLS Lab is used to define regions of interest (ROIs). Image processing strategies provide segments for laser capture microdissection. Protein extraction and tryptic digestion of small microdissected material is followed by proteomic analysis using PASEF.

Methods

Breast cancer tissue sections (12µm) were mounted on PEN (polyethylene naphthalate) membrane slides. Slides were coated with Norharmane matrix (7 mg/mL in CHCl3:MeOH, 2:1, v:v) using a TM-sprayer (HTX Technologies) and measured on a timsTOF fleX. After defining ROIs from lipid MALDI Imaging analysis in SCiLS lab, the data were processed in Matlab. Areas containing approximately 2000 cells were dissected from each segmented tumor subpopulation with a Leica LMD 7000 and directly transferred to reaction tubes for microextraction and tryptic digestion. Microextracted peptide samples were LC-separated and ran on the same TimsTOF fleX instrument using PASEF.

Results

SpatialOMx allows in-situ characterization of tissue subtypes. Lipid imaging on the timsTOF fleX from tumor sections was used to define ROIs by unsupervised segmentation. Intratumor heterogeneity was further analysed by in-depth proteomics of small microdissected (LMD) tissue pieces. In average, about 3500 protein IDs were obtained from the three microextractions. Interestingly, the proteomic profiles of the tumor subpopulation differed largely. The gene ontology terms “Biological regulation” and “Developmental processes” were underrepresented in subpopulations 1 and 2, respectively, while “cellular component organization” was overrepresented in tumor subpopulation 3.

Conclusions

The SpatialOMx workflow on the timsTOF flex provides guidance to specific regions of interest for in-depth proteomic analysis to characterize regiospecific molecular changes in detail.

References

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P111

SpatialOMx® utilizing MALDI-2 technology adds tissue context to traditional Omics data for in-depth investigation of regional cellular processes

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Topic: MS-Imaging

Introduction
Understanding disease often requires comprehensive knowledge about molecular processes at their spatial occurrence. Current approaches are not seldom limited by either, the analytical depth, or the lack of spatial information. SpatialOMx bridges this gap by providing deep Omics data that is connected to the tissue origin through MALDI Imaging. MALDI postionization (MALDI-2) helps to extend the detection range by intensity boosting for several analyte classes.

Methods
Fresh-frozen kidney sections of 10 µm thickness were used for MALDI Imaging analysis to study the distribution of lipids and metabolites on a dual source timsTOF fleX system (Bruker Daltonik GmbH). Data were acquired in positive and negative ion mode with and without MALDI-2 postionization. MALDI matrix for MS imaging included 9-aminoacridine (negative mode), DHB (positive mode, conventional MALDI) and MALDI-2 matrix (positive mode, MALDI-2 measurements).
PASEF empowered metabolomics and lipidomics data from a kidney extract were acquired using the ESI capability of the timsTOF fleX instrument in positive and negative ionization mode. Data quality was evaluated, and compounds annotated using MetaboScape® 2021 software to generate an Analyte List.
ESI and MALDI data were merged by application of SCiLSTM 2021a and MetaboScape® 2021 for automatic annotation of MALDI Imaging derived compounds.

Results
As a proof of concept, renal glomeruli served as small tissue regions which were defined in the MALDI Imaging data by spatial segmentation. H&E staining after MALDI Imaging provided histological confirmation. Compounds specifically localized in these regions were detected and automatically annotated based on the IDs generated from the Omics data. As an example, we identified PC (36:2) as being localized in glomeruli. MALDI-2 Imaging extended the detection range to increase the analytical depth and allowed for the visualization of e.g. cholesterol and vitamin E.

Conclusions
The SpatialOMx workflow allows for regiospecific, deep analysis of tissue samples.

P112

Commemorating the successful high-stringency identification of >90% of the human proteome

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Topic: Multi-omics and Systems Biology

Introduction
Human Proteome Project (HPP) was launched as the Human Proteome Organization (HUPO) initiative during the 9th HUPO Annual World Congress in 2010. HPP provides an international framework for scientific collaboration, data sharing and quality assurance, and strengthening the human proteome annotation. Over the last decade, HPP has initiated multiple partnerships, developed community accepted data interpretation guidelines, and undertaken re-analysis of the community-generated data. Here we present the first high-stringency HPP blueprint paralleling the HPP milestone of reaching the assembly of >90% of the human proteome, with high-stringency evidence. The timeline for this remarkable achievement will be available at https://hupo.org/Proteomics-Timeline.

Methods
HPP relies on the community-generated data to create the high stringency human proteome knowledgebase (KB). neXtProt as an HPP official HPP reference KB provides an annual update on the status of missing proteins through “HPP release”. Further analysis of the neXtProt HPP release between 2011 and 2020 provided an overview of the protein families that are highly amenable or resistant to the mass spectrometry detection, and trends on the identification of missing proteins per chromosome.

Results
Confident detection of the human proteome has risen consistently from 69.8% in 2011 to 90.4% (17,874 out of 19,773) in 2020. The rate at which proteins are detected has recently slowed, suggesting that identification of the remaining 9.6% (1899, excluding the dubious 577 neXtProt entries) proteins could be difficult. Protein entries were consistently updated in line with the HPP reference proteome fine-tuning. Olfactory receptor family constitutes the highest number of missing proteins. In contrast, Zinc-finger protein family has the largest number of discovered proteins since 2011.

Conclusion
The decadal analysis demonstrates the success of the community efforts on identifying the missing proteins. This effort will be crucial in understanding the hallmarks associated with human biology and diseases through high-throughput omics data.

P113

SwissLipids, connecting lipids to proteins

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Topic: Multi-omics and Systems Biology

Introduction

Lipids play fundamental roles in membrane formation, energy storage, and signaling. Analytical methodologies like mass-spectrometry (MS) provide a means to analyze lipidome composition, but the integration of lipidomics data with other 'omics datatypes requires prior knowledge of lipid biology – including metabolic networks and links to enzymes, transporters and other proteins. To facilitate this task we developed SwissLipids (www.swisslipids.org), an expert curated knowledge resource that connects lipids to proteins.

Methods

We capture knowledge of known lipids and their metabolism from the literature by annotating the structures in ChEBI (www.ebi.ac.uk/chebi/) and the corresponding reactions in Rhea, a resource of biochemical reactions built on ChEBI (www.rhea-db.org). To describe lipid metabolism in SwissLipids, we link the Rhea reactions to the corresponding enzymes using UniProt (www.uniprot.org). We use the curated knowledge of known lipids and their building blocks to generate a library of all possible lipid structures, which we arrange in a hierarchical classification that links MS analytes to structures. Each lipid in the library is mapped to a ChEBI class, and all lipid components are mapped to ChEBI.

Results

SwissLipids currently comprises more than 590,000 lipid structures belonging to over 500 ChEBI classes and over 5000 enzyme-reaction pairs. The SwissLipids hierarchical classification links lipid analytes to possible lipid structures and proteins like this:

PC(38:4) -> PC(18:0/20:4) -> PC(18:0/20:4(5Z,8Z,11Z,14Z)) -> PLA2G4A.

All data is freely available to browse, search and download through our website and API. An ID mapping service maps LIPID MAPS and HMDB identifiers to SwissLipids, ChEBI and UniProt.

Conclusions

SwissLipids (www.swisslipids.org) is a freely available expert-curated knowledge resource that helps integrate lipidomics data with other 'omics. Like SwissLipids, UniProt now uses Rhea to annotate enzyme

function, and we plan to leverage UniProtKB as the basis to build an enhanced SwissLipids v2.0 – eventually covering lipids from all taxa in UniProt.

P114

Human proteome in UniProt – A high-quality reference to support integrated multi-omics functional analyses in biomedical research

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Topic: Multi-omics and Systems Biology

Introduction:
The UniProt Knowledgebase (UniProtKB, at www.uniprot.org) is a reference resource of protein sequences and functional annotation. A major focus of UniProt is the human proteome, which combines expert curation of protein sequences, functions, and human variation to create a platform for the functional analysis of genomics, transcriptomics, proteomics, and more recently metabolomics data.

Methods:
The human proteome is mainly derived from coding sequences submitted to INSDC (www.insdc.org) and predictions from Ensembl (www.ensembl.org) that are reviewed and aligned to the reference genome. These sequences are enriched with isoform-specific, expert curated knowledge of protein function using community standard vocabularies including the Gene Ontology (www.geneontology.org/) and Rhea (www.rhea-db.org) for the description of biochemical transformations catalyzed by proteins (see poster 'Representing the human metabolome in UniProtKB using Rhea'). Natural variants curated from literature or from large scale datasets are linked to ClinVar (http://www.clinvar.com/). Amino acid modifications as well as proteomic data are also integrated and links to more than 100 other knowledge resources are provided.

Results:
There are currently 97,047 sequences within 75,004 non-redundant entries in the human reference proteome. 93.9% of the 20,383 reviewed human entries are functionally annotated with Gene Ontology terms while 3,410 are linked to small molecules described with ChEBI (www.ebi.ac.uk/chebi/) the Chemical Entities of Biological Interest ontology. 36,772 disease-associated and 11,664 functionally characterized variants are described. All data can be accessed via the UniProt website, REST API or SPARQL endpoint and downloaded in a variety of formats.

Conclusions:
UniProt provides researchers with a high quality, comprehensive, non-redundant and freely accessible expert curated human reference proteome enriched with machine readable functional annotation using community standard ontologies like GO, ChEBI and Rhea - providing a framework for integrated multi-omics analysis from genome through proteome to metabolome.

P115

Three-in-one simultaneous extraction of proteins, metabolites and lipids for multiomics

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Topic: Multi-omics and Systems Biology

Elucidation of complex molecular networks requires integrative analysis of molecular features and changes at different levels of information flow. Accordingly, high-throughput functional genomics tools such as transcriptomics, proteomics, metabolomics and lipidomics have emerged to enable system-wide investigations. Unfortunately, analysis of different types of biomolecules requires specific sample extraction procedures that often only cover a restricted type of biomolecules. Thus several sets of samples are needed for different molecules.

Here we adapted a bi-phasic fractionation method to extract proteins, metabolites and lipids from the same sample (3-in-1) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) multi-omics. To demonstrate utility of the new method, we used bacteria-primed Arabidopsis leaves to generate multi-omics datasets from the same sample.

In total, we were able to identify and quantify 2778 proteins, 1967 metabolites and 425 lipid species in single samples. The molecules were integrated into molecular networks that regulate plant immunity.

The results have shown the clear advantages of the new 3-in-1 multi-omics method, including sample conservation, high reproducibility and tight correlation between different types of biomolecules. The differential molecules are potential targets for engineering or breeding for enhancing plant defense.

P116

Multi-omics analysis of pancreatic cancer stem cells to investigate their metabolism

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Topic: Multi-omics and Systems Biology

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer-related death worldwide. The aggressiveness of this type of tumor has been related to the presence of a small subset of cells, called pancreatic cancer stem cells (PCSCs), which are responsible to drive tumorigenesis and disease recurrence. It is known that PCSCs possess peculiar metabolic properties that distinguish them from the bulk of the tumour and that confer an high resistance to conventional treatments. However, it is not yet fully demonstrated whether PCSCs preferentially rely on a more glycolytic or oxidative metabolism in comparison to differentiated tumour cells.

Methods: Panc1 PCSCs (at 2, 4, and 8 weeks subculture) and relative parental cells (P) were grown and collected. Briefly, a label free strategy based on SWATH-MS analysis was adopted to perform both proteomic and metabolomic comparison between PCSCs and P cells. Bioinformatic analyses were conducted to integrate the omics datasets and to detect the deregulated metabolic pathways.

Results: Peculiar metabolic differences between PCSCs and P at different weeks of culture were detected. Multi-omics analysis showed a downregulation of glycolysis/gluconeogenesis and pentose phosphate pathway in PCSCs as compare to P cells. Moreover, these pathways resulted more induced in PCSCs at 2 weeks as compared to 4 and 8 weeks. Instead, TCA cycle and OXPHOS were particularly induced in PCSCs as compare to P cells, mostly in PCSCs at 4 weeks of subculture.

Conclusions: The multi-omics data integration suggested progressively shift from glycolysis to oxidative metabolism of PCSCs during the time of culture. These findings have laid the foundation for further functional studies to clarify the comprehension of the metabolic features of PCSCs with the final aim to identify specific targets for the development of new effective therapeutic approaches.

P117

Protein context shapes the specificity of domain-peptide interactions in vivo

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Topic: Multi-omics and Systems Biology

Introduction: Protein-protein interactions (PPIs) between modular binding domains and their target peptide motifs are thought to largely depend on the intrinsic binding specificities of the domains. The large family of SRC Homology 3 (SH3) domains contribute to cellular processes via their ability to support such PPIs. While the intrinsic binding specificities of SH3 domains have been studied in vitro, whether each domain is necessary and sufficient to define PPI specificity in vivo is largely unknown.

Methods: We combined genome editing, deep mutagenesis scanning, PPI mapping, growth phenotyping and single cell imaging in yeast to identify SH3-dependent PPI networks and their associated phenotypes. In addition, we used SWATH MS in human cells and quantified SH3-dependent phase separation in vitro.

Results: By performing domain deletions, mutations, swappings and shufflings, we find that most SH3s do not autonomously dictate PPI specificity in vivo. We show that both the sequence of the SH3 host protein and the position of the SH3 domains within their host are critical for interaction specificity and for cellular processes such as endocytosis. We further validate these findings using a human multi-SH3 adaptor protein. We highlight that the protein context of SH3 domains also influences key biophysical processes such as phase separation.

Conclusions: Our work demonstrates the importance of the interplay between a modular PPI domain such as SH3 and its host protein in establishing specificity to wire PPI networks. Considering these complex interplays will be essential for understanding how protein networks are rewired during evolution and in the context of mutation-driven diseases such as cancer.

P118

ReactomeGSA - Efficient Multi-Omics Comparative Pathway Analysis

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Topic: Multi-omics and Systems Biology

Introduction: The continuous increase in public data offers the great opportunity to extend own analyses. This is often complicated by the use of different 'omics technologies and different species. Pathway analyses have emerged as a solution to this problem. Nevertheless, researchers still require considerable bioinformatics knowledge to perform such analyses. Here we present ReactomeGSA for comparative pathway analyses of multi-omics, cross-species datasets.

Methods: ReactomeGSA is integrated in Reactome's existing web interface and also accessible through the ReactomeGSA R Bioconductor package with explicit support for scRNA-seq data. Data from different species is mapped to a common pathway space. Public data from ExpressionAtlas and Single Cell ExpressionAtlas can be directly integrated in the analysis. ReactomeGSA thereby greatly reduces the technical barrier for multi-omics, cross-species, comparative pathway analyses.

Results: We used ReactomeGSA to characterise the role of B cells in anti-tumour immunity. We compared B cell rich and poor human cancer samples from five TCGA transcriptomics and two CPTAC proteomics studies. There, B cell-rich lung adenocarcinoma samples lack the otherwise present activation through NFkappaB. This may be linked to the presence of a specific subset of tumour associated IgG+ plasma cells that lack NFkappaB activation in single-cell RNAseq data from human melanoma.

Conclusions: ReactomeGSA can derive novel biomedical insights by integrating large multi-omics datasets.

P119

The Alternative Proteome in the Aged Heart Revealed by Multi-Omics

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Topic: Multi-omics and Systems Biology

Introduction
Alternative splicing (AS) patterns are known to be dynamically regulated during organismal aging. RNA-Seq data in particular have revealed widespread changes in the expression of AS mRNA transcripts in the aged hearts, but how these changes translate into the proteome is unknown. We hypothesize alternative splicing modulates protein structure and function by altering protein isoforms along cardiac-disease related pathways, and that these isoforms could be discovered using a multi-omics workflow (RNA-seq and proteomics).

Methods
We collected cardiac ventricles from young (12 wk) and old (78 wk) C57BL/6 mice (male and female). RNA and proteins were extracted from each tissue. The isolated RNA was subjected to deep RNA sequencing and at the same time, the proteins were processed for tandem mass tag labeled quantitative proteomics analysis on a Q-Exactive quadrupole orbitrap mass spectrometer. We used an in-house Python software (JCAST; https://github.com/ed-lau/jcast) to create sample-specific protein sequence databases from RNA sequencing datasets that contain the protein isoforms likely expressed. MS spectra were searched against the custom databases using Comet/Percolator to identify and quantify isoform expression.

Results and Conclusion
We integrated RNA-seq with proteomics into a multi-omics pipeline towards deep profiling of protein isoform expression. Preliminary results revealed >250 differentially expressed protein isoforms in the aged heart at 10% FDR, including known and undocumented protein isoforms and proteins playing important structural or functional roles in the heart (e.g. ACTS, BASI, and HTAI2). Functional annotation showed the differentially expressed proteins are enriched in key biological pathways including oxidative phosphorylation, metabolic process, translation, and cell signaling. The data show a clear dynamic modulation of AS and protein isoform expression in the aged hearts. In ongoing work, we are comparing the data to the analysis of cardiac hypertrophy models, in order to pinpoint common nodes in the proteins and pathways between aging and disease development.

P120

Towards functional assignment of the full platelet proteome and transcriptome

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Topic: Multi-omics and Systems Biology

Introduction:
Advances in platelet transcriptomics allow the prediction of the complete human platelet proteome.

Methods:
Bottom-up identified human platelet proteomes of six cohorts (5,5k unique proteins). Genome-wide quantified transcripts (57k RNAs) in human platelets and megakaryocytes from BLUEPRINT databases; cut-off values for relevant expression. Supervised assignment to 21 platelet function classes (PFC) for 20.4k protein plus RNA genes.

Results:
Proteome. We integrated the bottom-up proteomes of six cohorts, encompassing 5.5k unique proteins (3.6k with copy number), 90% being present in ≥2 databases. Guided by UniProt-KB, protein-coding genes were assigned to 21 protein function classes according to subcellular location and assumed function. Of these, 0.4% were not present in the transcriptome, whereas 5.8% did not have relevant expression (in majority Secretory proteins).

Transcriptome. The total genome-wide transcriptome of platelets and megakaryocytes was assessed at 20.4k RNA species which were all classified (72% protein-coding, 18% RNA genes and 9% pseudogenes). The (protein-encoding) transcript levels in platelets and megakaryocytes were strongly correlated ($R=0.75$, $p<0.0001$).

Comparison. For 3,629 proteins with copy numbers in platelets, transcript levels maximised but did not otherwise determine protein abundance. We compared PFC profiles of the identified platelet proteome and the remaining expected proteome (based on relevant transcripts), revealing the following. Over-represented in the identified proteome were the classes: Membrane receptors/channels as well as Cytoskeletal, Metabolic and Signalling proteins. Under-represented were: Mitochondrial, Nuclear, Proteasome and Transcription proteins. Equally present were: Endoplasmic reticulum and Other vesicular proteins.

Conclusions:

(i) The total platelet proteome is expected to be 13-14k, containing only part of the mitochondrial, nuclear and proteasomal proteins from megakaryocytes. (ii) The (protein-encoding) transcript levels in platelets and megakaryocytes are strongly correlated, while protein abundance and transcript levels in platelets are not correlated.

P121

Targeted Proteomics-Driven Computational Modeling of the Mouse Macrophage Toll-like Receptor Signaling Pathway

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Topic: Multi-omics and Systems Biology

Introduction
The Toll-like receptor (TLR) signaling pathway in macrophages is essential for the initiation of effective innate immune responses. Subtle variations in the concentration, timing, and molecular structure of the stimuli (e.g., lipopolysaccharide (LPS)) are known to affect TLR signaling and the resulting pathway dynamics. Tight regulation is essential to avoid acute tissue damage and chronic inflammation. Computational modeling can test mechanistic hypotheses about how regulation is achieved and why it sometimes fails, causing pathologies.

Methods
In this investigation, targeted mass spectrometry was used to measure the absolute abundance of 51 (phospho)proteins of the mouse macrophage TLR pathway (C57BL/6J BMDMs; basal and stimulated (10 nM LPS, 30 min)). Protein-protein association and dissociation rates were estimated using molecular dynamics.

Results
The (phospho)protein abundances spanned four orders of magnitude: from 1,400 to 11 million copies per cell. Abundance decreases were observed for proteins known to be targeted for degradation in response to TLR pathway activation. Association rate constants were estimated for 256 protein-protein interactions using high performance computing. The (phospho)protein absolute abundance values and protein-protein interaction rates are being used as parameters for TLR pathway simulations. Rule-based pathway modeling and simulation is being performed using the Simmune software suite. Phosphosite absolute quantification is being performed to produce time-course and dose-response data to be used for pathway model training, testing, and validation. Previously published proteomics, phosphoproteomics, and flow cytometry data are also being used.

Conclusions
Here, we describe how these various experimental data and computational techniques are being integrated into a strongly data driven model of TLR pathway regulation. This research was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH.

P122

Multi-Omics of platelets of pancreatic cancer reveals regulatory functions

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Topic: Multi-omics and Systems Biology

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers, with a 5-year survival below 8%. Remarkably, PDAC is traditionally associated with hypercoagulation, but molecular mechanisms underlying tumor/platelets interaction remain controversial. Since PDAC cells can activate platelets and change their RNA and protein content, we performed an integrative study investigating the biological processes of differentially spliced mRNAs, expressed mRNAs, miRNAs and proteins. To understand their “dangerous liaisons”, we used benign disease as a background noise correction for inflammatory signals.

Methods: Blood samples from 11 PDAC and 11 age- and sex-matched patients with benign pancreatic diseases were collected. Platelets were isolated for omics profiling of small RNAs, mRNAs and proteins. Results: Statistical comparison of PDAC vs benign platelets revealed 48, 981, 154, 1091 and 54 differentially expressed miRNAs (canonical and isomiRs), intron-spanning RNA, mRNAs and proteins, respectively. We then explored molecular mechanisms underlying potential changes from normal status platelets at miRNA, gene and protein level. Data showed significant changes at miRNA levels suggesting a clear miRNome response in platelets to external stimuli. Indeed, an integration of gene ontology analyses of miRNAs, intron-spanning RNAs, mRNAs and proteins data revealed regulation of RNA splicing, translational initiation and ribosome biogenesis in PDAC platelets. Interestingly, gene ontology mining of the PDAC platelets proteome revealed an enrichment on spliceosome and ribosomal proteins suggesting a clear de novo protein machinery that could response to external stimuli mainly regulated from miRNAs.

Conclusions: Our study demonstrates that the platelets transcriptome and proteome undergo significant alterations when comparing benign patients to PDAC.

Together, these biological networks provide novel insights in the regulatory mechanisms within blood platelets and should prompt future studies in larger cohorts of patients to validate key pathways in disease development. Additional research for the integration of furthers omics levels is warranted.

P123

Representing the human metabolome in UniProtKB using Rhea.

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Topic: Multi-omics and Systems Biology

Introduction: The UniProt Knowledgebase (UniProtKB, www.uniprot.org) is a reference resource of protein sequences and functional annotation that is widely used for functional analyses of genomic, transcriptomic, and proteomic data (see poster Human proteome in UniProt – A high-quality reference to support integrated multi-omics functional analyses in biomedical research). Here we describe work that extends the utility of UniProtKB to combined analyses that also include metabolomics.

Methods: We have undertaken a complete reannotation of all legacy enzyme data in UniProtKB, including human enzymes, using Rhea (www.rhea-db.org), a freely available resource of expert curated biochemical reactions described using the ChEBI ontology of small molecules (www.ebi.ac.uk/chebi/). As part of this work we are also incorporating all enzyme annotations from SwissLipids (www.swisslipids.org), a knowledgebase of lipids and their biology, into UniProt (see poster SwissLipids, connecting lipids to proteins). We have used this enhanced enzyme dataset as the basis to develop a range of new tools and services that allow UniProt users to map small molecule metabolites to proteins and thereby integrate metabolomics and proteomics.

Results: We have annotated about 3,000 distinct human enzymes with Rhea, creating 7,250 annotations including 3,289 unique reactions and featuring 3,382 small molecules from ChEBI. UniProt users can now search for chemical names, chemical structures or chemical classes (from the ChEBI ontology) using the UniProt website and APIs.

Conclusions: Rhea and ChEBI extend the utility of UniProtKB for integrated analyses of genomic, transcriptomic, proteomic and metabolomic data (see “Enzyme annotation in UniProtKB using Rhea”, Bioinformatics (2019), PMID:31688925). This is complemented by mapping of post-translational modifications to ChEBI (see poster Mapping PTM data from UniProtKB to ChEBI facilitates integration of proteomics and metabolomics). Current work focuses on completing annotation of the human metabolome in UniProtKB using Rhea, while future work will complete the reannotation of all small molecule ligands using ChEBI.

P124

Discovery of organelle membrane remodelling associated with CAV1/CAVIN1 in prostate cancer using integrative Protein and Lipid Organelle Profiling (iPLOP)

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Topic: Multi-omics and Systems Biology

Introduction:
Mammalian cells are compartmentalised into membrane bound organelles, where dysregulated membrane composition leads to loss of organelle homeostasis and may underlie chronic diseases. The cholesterol-binding protein caveolin-1 (CAV1) normally forms specialised caveolae structures at the plasma membrane with the co-factor protein cavin-1 (CAVIN1). However, CAV1 is expressed in advanced prostate cancer without CAVIN1, exemplified by PC3 cells. Ectopic expression of CAVIN1 in PC3 cells lead to caveola formation at the plasma membrane and attenuated prostate cancer progression in vivo (1). In addition to plasma membrane structure changes, PC3-CAVIN1 cells showed redistribution of cholesterol to intracellular compartments (2), therefore, we hypothesize that membrane remodelling modulated by CAVIN1 regulates oncogenic organelle function in prostate cancer.

Methods:
To enable comprehensive evaluation of organelle composition and proteo-lipid interactions, we developed an integrative Protein and Lipid Organelle Profiling (iPLOP) workflow which incorporates density gradient organelle fractionation with lipidomic (Dynamic MRM) and proteomic (DDA MS/MS) profiling. Here, we used iPLOP to discover organelle lipidomic changes associated with PC3-CAVIN1 cells compared to PC3-CONT cells, and then conducted validation using cell-based assays.

Results:
iPLOP workflow yielded two major findings which may underpin the reduced aggressiveness in PC3-CAVIN1 cells: reduced long unsaturated lipids in the mitochondria and endoplasmic reticulum (ER), and elevated lipid droplets. Lipid droplets, mitochondrial and ER membrane composition and function have been associated with cancer hallmarks (3). These results were functionally validated using cell-based assays.

Conclusions:
iPLOP allowed discovery of CAVIN1-induced organelle remodelling in prostate cancer. This unbiased organelle profiling method can facilitate discovery in other cell systems and disease models associated with membrane remodelling.

References:
1. Moon et al. 2014 Oncogene 33:3561-70.
2. Inder et al. 2012 Mol Cell Proteomics. 11:M111.012245
2. Molendijk et al. 2020 Molecular Omics. 17:6-18.

P125

Metabolomics from the first phase analysis to study the responses of stomata to changes in water content.

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Topic: Multi-omics and Systems Biology

Earlier studies have shown that the stomatal movements in plants will be affected in the process of "peristomal transpiration". This concerns understanding the difference in water potential interacting inside and outside the leaf during a growing state. A regular scallion, which became a sample for this study indicated that the regular presence of H20 and light did not significantly affect the scallions firmness, visual quality, color, or chlorophyll level and carotenoid content within an average period of three to five days. The goal of this study aims to understand how alterations in the nutrients of water (mainly for hydroponic treatments in plants) have a growth promotion factor to the quality of plants. In this case, the application of metabolomics becomes a first phase experimental study to navigate the responses of changing the water status for scallion growth. The first phase results show that the construction of stomata in scallions had played a critical role in regulating gas exchanges and the photosynthesis-conducting chloroplasts. Clarity on these matters promises to challenge metabolomics modelers to characterize these interactions, and additionally identify validating hydroactive feedback mechanisms such as the pH content value in the water status.

P126

A real-time search engine for active sequence acquisition control

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Topic: New Technologies – Mass Spectrometry

Introduction: Recently we introduced PASER (PArallel database Search Engine in Real-time) which performs a database search in parallel with the 120Hz sequencing speed of the timsTOF Pro mass spectrometer. PASER was used with a high performance IP2-GPU search engine and completed simultaneous and uncompromised database search at mass spectrometry spectra acquisition time scale. Real-time search results are particularly empowering when they can inform the progression of the data acquisition queue, saving the loss of precious samples, acquisition time and delaying instrument acquisition until a pre-determined metric is qualified.

Methods: The mass spectrometer was a timsTOF Pro (Bruker) equipped with a nanoElute or Evosep and gradients from 3.6 mins to 90 mins were used. The sample was K562 (Promega) proteolytic digest. PASER box was a GPU enabled PC which performs the real-time search and information transfer. Using a “if then that” logic, a user defined metric (number of protein or peptide IDs) is used to inform the Hystar (Bruker) sequence acquisition software.

Results: After data acquisition was completed so were the search results, where if the identification of ≥3500 proteins and ≥16000 unique peptide observations in a 21min gradient were achieved then the sequence queue progressed. We did this in a progressive dilution series from 200ng to 5ng, where the qualifier was not met at loads less than 25ng and acquisition queue halted.

Conclusions: Real-time search results now enable intelligent acquisition sequences where user defined logic determine sequence progression.

P127

Analysis of human cell digests using a high-throughput SWATH® Acquisition and cloud-based data processing solution

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[1]*SCIEX*

Topic: New Technologies – Mass Spectrometry

Introduction: New data processing solutions are needed to match the speed and scale at which SWATH Acquisition results are collected. In this study, the OneOmics™ Suite, a cloud-based proteomics solution, was used to quantitate and compare protein expression differences across six human cell digests. The cell digest proteins were also quantitated using two distinct microflow LC gradients, a 20 min gradient and a 45 min gradient, to evaluate impacts of shortening the LC method.

Methods: Whole cell lysates (HEK293, Hela, MCF-7, A549 and MG132-treated Hela and MG132-treated HEK293) were digested with trypsin and loaded at 3-5 µg total protein per injection. A NanoLC™ 425 system was used for microflow chromatography at two gradient lengths. SWATH Acquisition data was collected using the OptiFlow® Ion Source on the TripleTOF® 6600+ System. All SWATH Acquisition Data was processed in SCIEX Cloud using the OneOmics Suite.

Results: Shortening the microflow LC gradient from 45 to 20 min resulted in similar numbers of proteins quantified per digest at 1% FDR and 20% CV and no significant reduction in quantitative accuracy. More peptides per protein at 1% FDR and 20% CV were quantified with the 45 min gradient. Using the Browser and Biological Review tools in the OneOmics Suite, expression profiles of individual proteins could be compared across the cell lines, along with enrichment of ontologies and biological pathways.

Conclusions: The OneOmics Suite enabled fast processing and results interrogation of SWATH Acquisition data collected for six distinct cell digests at two microflow gradient lengths. The numbers of proteins quantified were very similar between the 20 and 45 min gradients, with no significant degradation of quantitative accuracy.

P128

Mass Spectrometry Characterization of Peptides Through Acetone Precipitation

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Topic: New Technologies – Mass Spectrometry

Introduction: Precipitation is a classic approach that can be used to fractionate, concentrate, or purify proteins from complex biological systems. With organic solvent precipitation, low molecular weight (LMW) proteins are generally less susceptible to aggregation in organic solvent and remain in the supernatant. The objective of this work is to optimize acetone precipitation of LMW proteins and peptides and determine the low mass limit for solvent-based protein precipitation. We aim to answer whether small peptides can be precipitated in acetone, and to what extend the peptide sequence influences recovery.

Methods: Trypsin-digested salmon and yeast were used to generate a mixture of low molecular weight protein fragments (<5 kDa). The sample was precipitated by adding acetone with inclusion of various concentrations of salts. The pellet was isolated from the supernatant and subjected to analysis by HPLC with UV quantitation and LC-MS/MS for identification and characterization of proteins/ peptides recovered in the resulting fractions.

Results: The results indicate a strong dependence on the type of salt in the sample. The combination of $ZnSO_4$ with high volume of acetone provides near quantitative recovery of all peptides down to 2 kDa, and continues to exceed 90% yield for peptides at a molecular weight of 1 kDa. Also, MS characterization of peptides demonstrates that except the size of peptides, there are no other specific trends leading to influence the recovery of the peptides following by optimized precipitation.

Conclusion: The proposed protocol is applicable to the precipitation of LMW peptides ahead of MS analysis which are important targets for biomarker discovery and peptidomics analysis.

P129

High-precision ion mobility calibration for dia-PASEF analysis increases proteome coverage for high throughput 4D-Proteomics

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Topic: New Technologies – Mass Spectrometry

Introduction:
Data-independent acquisition (DIA) facilitates reproducible and accurate protein identification and quantification across large sample cohorts by using wide selection windows to ensure fragmentation of all precursor ions. Trapped ion mobility spectrometry (TIMS) provides an additional dimension of separation for complex proteomics samples without a loss in sensitivity. TIMS based DIA has recently become available on the timsTOF Pro as a new acquisition mode, called dia-PASEF. A novel high-precision ion mobility calibration workflow was designed in Spectronaut 14 to improve ion mobility based DIA data. Here, we combine Bruker's dia-PASEF technology with this workflow to investigate its utility for complex proteomics samples using different gradient lengths.

Methods:
A tryptic digest of yeast was analyzed by a nanoElute LC coupled to a timsTOF Pro mass spectrometer (both Bruker Daltonics). dia-PASEF data was acquired for 30, 60 and 90-min gradients using an optimized dia-PASEF scheme and processed using Spectronaut with 1% FDR at peptide and protein level. A comprehensive IMS-enhanced sample-specific library was generated from fractionated DDA PASEF runs using Spectronaut's Pulsar database search engine with 1% FDR at PSM, peptide and protein level.

Results:
With 4410 protein groups and 45,957 peptides identified from 90-min gradient dia-PASEF runs of yeast digest, targeted 4-dimensional extraction based on high-precision IMS calibration increased identification rates on average by 20% when compared to 3-dimensional extraction without making use of ion mobility. Remarkably, this translates into 86% coverage of all proteins in our comprehensive library, highlighting the exceptional sensitivity of the timsTOF Pro. By using shorter 30-min gradients, we were still able to identify more than 4000 protein groups covering 79% of proteins in the library.

Conclusions:
Our results highlight that the combination of shorter gradients with high-precision ion mobility calibration dia-PASEF analysis using Spectronaut achieves deep protein coverage and is ideally suited for high-throughput proteomic profiling.

P130

Ion mobility separation in a TIMS-TOF PASEF acquisition method decreases spectral complexity

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Topic: New Technologies – Mass Spectrometry

Introduction: In bottom-up mass spectrometry based proteomics of complex samples, deep proteome coverage is often limited by peptide cofragmentation resulting in complex fragment ion spectra with signals from multiple peptides. This issue can be addressed by additional separation, either off-line or on-line. Trapped Ion Mobility Spectrometry (TIMS) separates ions on-line by their collisional cross section and has been integrated into Parallel Accumulation-Serial Fragmentation (PASEF) workflows to increase ion beam coverage without reducing sensitivity.[1] Here, we quantify the effect of ion mobility (IM) separation on cofragmentation of peptide precursors using experimental data and its effect on increasing peptide and protein identification rates.

Methods: LC-MS-IM features were identified by MaxQuant from a tryptic HeLa cell digest, acquired on a timsTOF Pro (Bruker) instrument in PASEF data dependent acquisition (DDA) mode (PXD010012).[1] To evaluate the separation power of TIMS IM we compared LC-MS-IM feature overlap and spectral complexity against the LC-MS space.

Results: IM separation increased the rate of non-overlapping features 9-fold, from 5% to 46%, yielding more pure precursors for MS2 analysis. The number of overlaps per feature are reduced 6-fold due to an overall reduction to precursor density. In DDA, with isolation windows of 2-3 Da, IM increases the number of frames without cofragmentation 4-fold and increases the precursor ion fraction (PIF) 4.5 fold, resulting in an estimated 1.7-fold increase in peptide spectral matches. In 25 Da windows, typical of a Data Independent Acquisition experiment, IM reduces the degree of cofragmentation between 1.7 and 5.7-fold depending on post-acquisition extraction.

Conclusions: TIMS IM separation had a dramatic effect on the separation power resulting in cleaner spectra which contributes to increasing peptide identification rates and a deeper proteome coverage.

1. Meier, F. et al. Mol. Cell. Proteomics (2018) doi:10.1074/mcp.TIR118.000900.

P131

A supervised learning approach to select the collision energy maximizing peptide fragmentation in ion mobility-mass spectrometry

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Topic: New Technologies – Mass Spectrometry

Introduction. The ability to identify peptides and proteins in tandem mass spectrometry is directly linked to the level of ion fragmentation achieved. Typically, the better the fragmentation, the higher the signal-to-noise ratio will be in the tandem mass spectra, which generally facilitates the identification of their corresponding peptides. Ion properties such as the mass-to-charge ratio (m/z) and charge state impact the level of collision energy needed to achieve an optimal fragmentation. Nevertheless, several mass spectrometry instruments do not make use of such information to determine the optimal collision energy during their analysis.

Methods. Herein, we present a supervised learning algorithm that uses measurements from ion mobility-mass spectrometry and a collision energy value to predict the relative fragmentation of precursor ions. Specifically, we designed an artificial neural network using features such as m/z, charge state, and ion mobility coefficient (1/K0) to predict relative precursor ion fragmentation for a given collision energy value on a Bruker timsTOF Pro. We then use this neural network to determine the optimal collision energy.

Results. Using a cross-validation strategy, we show that our algorithm can accurately predict relative ion fragmentation ($R^2 = 0.77$). We demonstrate that while m/z is the main criteria that should be considered for optimal collision energy selection, additional ion properties can further guide the quality of such a choice. We also show that our algorithm's running time allows its execution in real-time during the high-speed data acquisition of the timsTOF Pro instrument.

Conclusions. Finally, we demonstrate that not only can the relative fragmentation level of a peptide ion be predicted using a neural network, but that our algorithm can be used to optimize collision energy selection during mass spectrometry analysis to maximize fragmentation. By enhancing fragmentation, our approach will increase signal-to-noise ratio in tandem mass spectra and therefore facilitate peptide identification.

P132

EasyPep Sample Preparation Technology for Rapid and Efficient Mass Spectrometry-based Proteomics

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Topic: New Technologies – Mass Spectrometry

Introduction
Advances in mass spectrometry (MS) instrumentation have enabled routine analysis of complex protein samples. However, protein sample preparation for mass spectrometry still largely lacks cohesive standardization, which generally leads to inconsistent and irreproducible analyses. Recently, we expanded our EasyPep™ sample preparation technology by introducing two new formats – a large-scale (Maxi) sample preparation kit to streamline the analysis of protein post-translational modifications and a 96-well plate format kit to enable high throughput and automation. Here, we describe sample type-specific examples to highlight the unique features of each format for different sample types and applications.

Methods
Several cell and tissue types, including FFPE and plasma samples, were processed using our standardized EasyPep sample preparation procedures according to the format-specific procedures for both label-free and Tandem Mass Tag™ (TMT™) reagent labeling experiments. Peptide yields were assessed using Pierce™ Quantitative Colorimetric Peptide Assay prior to LC-MS analysis using Thermo Scientific™ Q-Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Results
Our optimized chemistry enabled efficient and reproducible processing of cultured mammalian cells and tissues significantly reduced both hands-on and total sample processing time. We were able to routinely obtain 10-20% higher protein and peptide identifications with lower missed cleavages than other common workflows. In addition, we demonstrate that EasyPep sample prep chemistry and cleanup is fully compatible with TMT or TMTpro reagent multiplexing for processing and quantitative analysis of large numbers of samples within a few days. Three operation modes including centrifugation, vacuum, and positive pressure were evaluated and optimized for 96-well EasyPep sample preparation plate. Both vacuum and centrifugation modes were evaluated for the larger Maxi column format. We have also adapted EasyPep 96 sample preparation protocol to a fully automated workflow using a Hamilton™ STARlet™ automated liquid handling system.

Conclusion
Optimized workflow for quick and efficient sample preparation for discovery and quantitative proteomics.

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Optimized informatic pipeline for the automated execution of SureQuant workflow on Orbitrap mass spectrometers

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Topic: New Technologies – Mass Spectrometry

Introduction

The SureQuantTM methodology has recently been implemented natively in the instrument control software of Orbitrap-based mass spectrometers. It uses spiked-in internal standards (IS) to dynamically control the acquisition process and to maximize its productivity. In addition to the resulting enhanced data quality and expanded scale of targeted experiments, the technique exhibits an improved usability and robustness, owing to its independence from time-scheduling acquisition, and therefore high potential for automation. In order to progress further towards a turnkey solution, several informatic developments have been conducted to optimize the preparation of SureQuant method and the processing of generated data.

Methods

The SureQuant method has been implemented in the instrument control software of Thermo Scientific™ Orbitrap Exploris™ 480 and Tribrid™ mass spectrometers. New data processing functionalities have been implemented in Skyline software to better match the specificities of SureQuant data. Specific Proteome Discoverer nodes have been developed to contribute to the supporting informatic pipeline.

Results

In order to deal with the more complex connecting process between analytes and MS2 scans in SureQuant raw files, a new scan filtering strategy has been implemented in Skyline, better exploiting the analyte-derived information stored in the raw files. In addition, the Skyline integration routines have been revisited to more properly manage the non-systematically paired measurement of IS and corresponding endogenous peptides and maintain good quantification accuracy. In parallel, a new peak boundaries definition process has been fully optimized and implemented in a Proteome Discover node to be used in conjunction with Skyline, avoiding the need for manual reviewing/correction of peak integration. Another Proteome Discover node has been developed to better automate the development and deployment of SureQuant assays through “one-click” method generation.

Conclusions

Several informatics developments have been conducted to minimize/preclude any manual intervention in the SureQuant workflow and progress towards its full automation.

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Proximity Labeling Proteomics of Mammalian Lysosomes

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Topic: New Technologies – Mass Spectrometry

Introduction: Autophagy-lysosomal pathways are critical to cell survival by recycling misfolded proteins and damaged organelles. Lysosomes frequently make contact with other organelles, but these transient and dynamic interactions can only be captured by live cell microscopy currently, one interaction at a time. Here, we developed two novel proximity labeling proteomic strategies to characterize the dynamic lysosomal interactome both in vitro (human neuron culture) and in vivo (fixed mouse brain tissues).

Methods: The bait protein, lysosomal-associated membrane protein1 (LAMP1), was fused with a peroxidase to create a transient 10-20 nm radius to label proteins on and surrounding the lysosome membrane. The labeling events in both human neurons and mouse brain slices can be activated by phenol biotin incubation and brief H2O2 treatment. Biotinylated proteins were then enriched by streptavidin magnetic beads followed by on-beads digestion, peptide desalting, LC-MS/MS analysis, and data analysis.

Results: Key lysosomal membrane proteins were identified and quantified such as LAMP1 (bait), LAMP2, LAMPTOR complex proteins, TMEM106B, CLCN5, PIP4P2, et al. The proximity-labeling proteomic results demonstrated specific and complementary identification of lysosomal membrane and membrane-interacting proteins between human neurons in culture and fixed mouse brain tissues. Lysosomal localization of labeled prey proteins was validated by fluorescence microscopy. We also conducted a comprehensive investigation and optimization of the key parameters in the entire proximity labeling proteomic workflow, greatly improving the accuracy, specificity, and reproducibility of protein quantification in proximity labeling techniques.

Conclusions: In summary, we developed novel proteomics strategies to characterize the lysosomal membrane and membrane interacting proteins both in vitro and in vivo, which provide unique tools to study lysosome dysfunction involved in various human diseases.

P135

Less is more: Avoiding artificial modifications in proteomic sample preparation for pharmaceutical and clinical applications

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Topic: New Technologies – Mass Spectrometry

Introduction: Within the last years, mass spectrometry-based protein analyses emerged as a powerful analytical tool in various pharmaceutical and clinical applications. While LC-MS instrumentation is continuously evolving, irreproducible and tedious home-brew sample preparation workflows are still prevalent. To simplify and standardize sample preparation, we developed an extensively optimized workflow, providing a comprehensive solution for various sample types including body fluids and therapeutic proteins. Based on the in-StageTip (iST) sample preparation, we combined minimum time expenditure with excellent reproducibility, particularly focusing on prevention of artificial modifications and increasing protein identifications.

Methods: LC-MS sample preparation was performed for human plasma, the NIST mAB reference standard and selected single proteins comparing the newly developed iST-BCT workflow against common workflows, including the regular iST protocol, a home-brew urea workflow and commercially available kits. The iST-BCT workflow is based on the iST-technology and optimized for pharmaceutical and clinical applications. By employing a novel detergent that is stable at alkaline pH, lysis and digestion is performed at low pH to suppress artificial modifications. Additionally, alkylation efficiency was improved by using a stronger reducing agent and by optimizing the buffer composition. Subsequent MS runs were performed in DDA mode on a timsTOF Pro or a LTQ-Orbitrap XL and analyzed with the MaxQuant software.

Results: We demonstrate that our novel iST-BCT workflow enhances the performance in comparison to the regular iST protocol and all other tested sample preparation solutions when processing body fluids such as plasma or therapeutic proteins such as the NIST mAB reference. Notably, iST-BCT reduces artificial modifications, such as oxidation and deamidation, by up to 60 % while boosting alkylation efficiency up to 50 % compared to the iST protocol.

Conclusion: With this novel iST-BCT workflow we set a new standard in pharmaceutical and clinical applications by simplifying and standardizing sample preparation.

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Extending Capabilities of Real-Time Database Search on the Orbitrap Eclipse Tribrid Mass Spectrometer for Multiplexed Proteomics.

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Topic: New Technologies – Mass Spectrometry

Introduction
The Real-Time Search (RTS) feature implemented on the Orbitrap Eclipse grants the ability to generate peptide spectrum matches (PSMs) from MS2 spectra during acquisition via database search. Only upon confident identifications, the instrument may be triggered to proceed with SPS-MS3. In this work, we evaluated recent improvements to RTS based TMT quantitation workflows including: 1) Close-out to prevent acquisition of excess MS3s for proteins which have already reached a sufficient depth of quantitative sampling, 2) online false discovery rate (FDR) assessments to predicate MS3 events on real-time PSM-level FDR estimates.

Methods
Thermo ScientificTM PierceTM TMT11plex Yeast Digest Standard or TMTpro 16 plex 2 proteome (human and E.coli) samples were analyzed using a Thermo ScientificTM Easy-nLCTM 1000 with 50 cm EASY-SprayTM columns coupled to an Orbitrap Eclipse Tribrid mass spectrometer. Data acquisition was carried out on the Tune 3.4 software. MS2 spectra were analyzed during RTS by the Comet search algorithm, and post-acquisition by Sequest HT in Thermo ScientificTM Proteome DiscovererTM Software 2.5.

Results
The RTS-MS3 workflow increased quantification accuracy and the number of quantified features compared to SPS MS3 methods, with the RTS “TMT mode” option (RTS-informed selection of TMT containing fragments for SPS isolation) outperforming the “trigger only” option. Enabling the Close-Out feature maintained quantitative accuracy while further boosting the improvement in quantified proteins. When the RTS FDR-filtering option was enabled, more MS3 spectra were collected compared to a heuristic score cutoff, and the signal-to-noise ratio of reporter ions was enhanced. The proportion of PSMs with greater than 90% SPS window placements matching identified fragment ions increased by 20% on average when the RTS-FDR threshold was applied.

Conclusions
The new features in the RTS-MS3 workflow extend its capabilities for TMT quantitation. Improvements in numbers of quantified proteins and peptides were observed.

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Reproducible targeted peptide profiling using highly multiplexed MRM assays

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Topic: New Technologies – Mass Spectrometry

MRM analysis for targeted protein quantification studies is driven by the renowned sensitivity and selectivity of QQQ-based MS systems. But quantitative robustness remains key, to ensure both large and small biological changes are accurately measured across large sample cohorts. Biological matrices also require the LC-MS/MS system to have high sensitivity as well as wide linear dynamic range.

Methods: A study was performed in simple and complex matrices to test the performance of the SCIEX Triple Quad™ 7500 LC-MS/MS System for large scale targeted peptide quantification. Methods with up to 4000 MRM transitions were run using Scheduled MRM Algorithm using microflow chromatography (5 and 30 min gradients) and the quantitative reproducibility was evaluated.

Results: The average retention time %RSD observed for the 10-15 replicates analyzed using each method was 0.13% and 0.2% for both the 5 and 30 min gradients. This allowed the dwell times to remain above 5 msec even for the 4000 MRM methods.

The effect of higher numbers of MRM transitions on the reproducibility of 50 peptide MRMs (3 protein digests in buffer) across 15 replicates on the SCIEX 7500 System was assessed using microflow chromatography (30 min gradient). High reproducibility was observed for methods with up to 4000 MRMs per method, with ~85-90% of peptides showing %CV<5%. A similar study was then performed with the same 3 protein digests spiked in complex matrix with 10 replicates. Again, high reproducibility of up to 3000 MRMs was achieved, with 90% of peptides having %CV <4.5%.

Conclusions: Sensitivity and quantitative reproducibility of the SCIEX Triple Quad 7500 System enables very good peak reproducibility to be observed even with very high numbers of MRM transitions in a single assay. Coupled with highly reproducible microflow chromatography, this LC-MS/MS system provides a powerful tool for large scale targeted peptide quantification studies.

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Optimisation and validation of a robust sputum proteomics protocol for clinical proteomics

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Topic: New Technologies – Mass Spectrometry

While sputum proteomics have been used to discover biomarkers and pathogenic mechanisms for respiratory diseases such as asthma, the lack of cohort replication and validation limit the applicability of these results. Generally, validated high throughput, robust sample processing method is essential for clinical proteomics in diverse matrices. To address the gap for sputum proteomics, the current study aimed to establish an optimal, robust high throughput method and to characterise detection limits and reproducibility.

Sputum viscosity and the presence of large mucins may contribute to variability. Deglycosylation of sputum sample did not alter the protein identification, hence a standard trypsin digestion and C18 clean up method on the Bravo liquid handler was selected. To validate the linearity and reproducibility of the method, an internal standard protein (chicken ovalbumin) was spiked into a sputum matrix (pooled from three volunteers) at 10, 50, 100 and 250 ng/ml and each split into 3 technical replicates. After high throughput processing, peptides were analysed on a Q Exactive Plus with Ultimate 3000 RSLC using a 120 min gradient. MaxQuant was used for database searching and protein intensities were extracted for external statistical analyses included one-way ANOVA, %CV and Bland Altman test.

The current protocol identified 1150 ±50 proteins at a false discovery rate (FDR) of 1%, and a CV of 4.4. By using a spike-in protein, assay linearity was demonstrated by and R2 value of 0.99 and the detection limit was determined to be 5 ng/mL for chicken ovalbumin. The functions of the proteome were relevant to asthma, including neutrophil regulation, interleukin 5 pathway, apoptosis, phagocytosis and bacterial invasion of epithelial cells.

Validation of assay linearity and limit of detection using a spike-in protein confirms the high throughput sputum proteomics method to be highly robust and reproducible. This procedure will facilitate clinical and translational sputum proteomics.

P139

Photo-cleavable Surfactant Enabled Extracellular Matrix Proteomics

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Topic: New Technologies – Mass Spectrometry

Introduction: The dysregulation of extracellular matrix (ECM) proteins and post-translational modifications (PTMs) directly contributes to diseases such as cancer. However, ECM proteomics remains extremely challenging due to the insoluble nature of the ECM. Herein, enabled by effective solubilization of ECM proteins using our recently developed photocleavable surfactant, Azo, we have developed an ECM proteomic strategy that allows fast tissue decellularization, efficient extraction and enrichment of ECM proteins, and rapid digestion prior to reversed phase liquid chromatography (RPLC)-MS analysis.

Methods: Mouse mammary tumor tissue was decellularized in 2% Triton-100 and the ECM was extracted in Azo, an anionic, photo-cleavable surfactant. Proteins were reduced, alkylated and digested with trypsin for two hours. After quenching, UV-irradiation was utilized to rapidly degrade the surfactant prior to reversed-phase liquid chromatography (RPLC). For the 2D approach, peptides were first fractionated offline using high-pH RPLC. Electrospray ionization (ESI) was coupled to Bruker Maxis QTOF II or Impact II for MS/MS.

Results: Azo improved ECM extraction and trypsin digestion conditions allowing elimination of traditionally used chemical digestion methods, like cyanogen bromide or urea, that require time consuming sample clean-up. We identified 280 unique gene products in a high-throughput, facile manner. An abbreviated list of ECM proteins was created as a useful tool for researchers to identify targets of interest for future studies. We identified a diverse range of ECM PTMs including hydroxylation, acetylation, phosphorylation, and glycosylation. In total, 585 ECM PTM sites were identified. Significantly we observed a dramatic increase in PTM identifications (3,623 total) with the 2D approach.

Conclusions: We developed a novel, robust, and high-throughput strategy to better characterize ECM proteins, which are highly desirable targets for the development of therapeutics. This Azo-enabled ECM proteomics strategy will streamline the analysis of ECM proteins and promote the study of ECM biology.

P140

diaPASEF: Bottom-up proteomics with near optimal ion usage

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Topic: New Technologies – Mass Spectrometry

Data-independent acquisition (DIA) methods have become increasingly popular due to high quantitative accuracy and reproducibility. However, the typical sequential acquisition of fragment ion spectra limits the ion sampling efficiency to only 1-3%. Previously, we have demonstrated that synchronizing trapped ion mobility spectrometry (TIMS) and precursor ion selection in a QTOF mass spectrometer increases MS/MS sequencing rates more than ten-fold in a novel acquisition mode termed PASEF. Here, we applied the PASEF principle to DIA and investigate its performance in typical proteomics applications.

LC-TIMS-MS experiments of tryptic proteome digests were performed on a timsTOF Pro mass spectrometer (Bruker Daltonics). Electrosprayed ions were accumulated for 100ms in the first TIMS analyzer, while, in parallel, mobility analyzing another batch of ions in a second TIMS analyzer. We stepped the quadrupole isolation window as a function of the TIMS release time to cover the precursor-mass-mobility range of interest. Data extraction was performed with the Mobi-DIK software.

We demonstrate close to 100% ion usage for low-complexity samples by defining a single diagonal diaPASEF scan lines in the m/z vs ion mobility plane. In whole-cell proteome samples, we defined multiple diaPASEF scan lines to reduce spectral complexity, however, the detected fragment ion current was still about a factor of five higher as compared with conventional DIA and similar precursor selectivity. Library-based analysis of 200ng HeLa digest identified on average 56,000 peptides and 7,000 proteins in triplicate 120min runs. The data completeness was 96%, indicating a very high reproducibility. We investigate the performance of diaPASEF in terms of sensitivity. In total, we quantified over 4,000 proteins of only 10ng HeLa digest. We also anticipate that tuning diaPASEF to very fast cycle times <1s will greatly benefit short gradient analysis.

diaPASEF makes efficient usage of incoming Ions resulting in high sensitivity and comprehensive proteome coverage in single runs.

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High throughput proteomics - Application of dia-PASEF for short gradients

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Topic: New Technologies – Mass Spectrometry

Introduction
Data-independent acquisition (DIA) promises reproducible and accurate protein identification and quantification across large sample cohorts by using wide selection windows to ensure that all precursor ions are fragmented in every sample. Ion mobility separation provides an additional dimension for separation of complex proteomics samples, that can also be used for alignment of precursor and fragment information. Here, we combine the PASEF technology (Meier et al., 2018) with a DIA approach and investigate the potential for complex proteomics samples using short gradients.

Methods
An in-house tryptic digest of HeLa was analyzed by coupling an Evosep One system (Evosep Biosystems) to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro, Bruker Daltonics). A dia-PASEF scheme optimized for the short gradient methods has been used for targeting +2 and +3 ions in a three-window method covering an m/z range from 400 to 1000 with a total cycle time of 900ms per dia-PASEF cycle. Data processing was done using Spectronaut 14 (Biognosys).

Results
DIA workflows rely on spectral libraries for the correlation of quantitative data from fragment ion spectra with peptide identifications. We used PASEF in DDA mode and fractionated samples to assemble the resource-specific library using Spectronaut software. The library comprised 8,381 protein groups and 93,301 peptide sequences in ~10h of data acquisition. We applied the hybrid library approach supported in Spectronaut for shorter gradient data by combining the resource-specific library with a project-specific library. This workflow allows keeping retention time precision of the shorter gradients for targeted data extraction. Using the comprehensive libraries, we could identify and quantify on average 5,204 protein groups and 39,936 peptide sequences using 60 SPD method at a 1% FDR.

Conclusion
The dia-PASEF method in combination with short gradients enables high sample throughput without sacrificing depth and quantitative accuracy of analysis.

P142

A methodological approach to increase protein identifications in exhaled breath condensate

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Topic: New Technologies – Mass Spectrometry

Introduction: Exhaled breath condensate (EBC) is a promising target for the biomarkers detection of socially significant lung pathologies, including cancer. It contains thousands of compounds reflecting lung metabolism. Protein and peptide EBC biomarkers are of particular interest and require high-sensitive analytical methods, such as high-resolution mass-spectrometry. Currently, the lack of standardized EBC collection and sample preparation protocols still remains the major obstacle to widespread clinical use of EBC analysis. Herein, in order to reveal the optimal conditions for the high-yield identification of EBC proteins, a particular sampling modification was proposed.

Methods: EBC samples from healthy controls and lung cancer patients were collected with RTube® device (Respiratory Research, Inc., Charlottesville, VA). After EBC removement, the collecting tube was rinsed with 1 ml of methanol. EBC and methanol fractions were analyzed by poly-acrylamide gel electrophoresis (PAGE), and peptides obtained after tryptic digestion were analyzed by LC-ESI-MS/MS using a nano-LC Agilent-1100 system coupled to 7T LTQFT Ultra tandem high resolution mass-spectrometer (the equipment of the Center for Science and Technology at the IBCF of RAS “New materials and new technologies”).

Results: Both PAGE and MS studies confirmed that methanol fraction was more enriched with proteins (~1.5 fold) and peptides (~2 fold) than EBC. About 30 proteins were found to be the most common in different samples, including keratins, desmoplakin, desmoglein-1, galectin-7 and others. The additional methanol rinsing essentially reduces the protein sorption at the inner surface of the collecting device. The analysis of both EBC and methanol fraction provided the essential improvement in EBC peptide and protein identifications.

Conclusions: The proposed methodological improvement is extremely relevant for development of reliable procedures for EBC proteome and peptidome analysis, which may provide highly important tool for timely noninvasive diagnostics of numerous socially significant diseases, including lung cancer. The research was supported by RFBR grant #18-29-09158MK.

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Proteomic workflows for high quality quantitative proteome and PTM analysis of clinically relevant samples from FFPE archives

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Topic: New Technologies – Mass Spectrometry

Introduction: Well-characterized archival FFPE tissues are of great value for prospective biomarker discovery studies within cancer research. Due to the known challenges of working with FFPE tissues in proteomics, well-established protocols that offer high-throughput and good reproducibility are essential.

Methods: Therefore, we developed a workflow implementing efficient paraffin removal and protein extraction from FFPE tissues, followed by digestion using suspension trapping (S-trap). To gain proteomic depth, the protocol was combined with isobaric labeling and applied to lung adenocarcinoma patient samples. The peptides eluted from the S-trap were labeled with TMT without any desalting step in between.

Results: In total, 8,163 proteins were commonly quantified across all channels, and proteins related to clinical outcome and histopathological subtype were detected. Since acetylation is known to play a major role in cancer development, an on-filter acetylation protocol was developed for studying endogenous lysine acetylation in FFPE tissues. Our method allows identification and localization of lysine acetylation and quantitative comparisons between samples. We could identify 1,839 acetylated peptides belonging to 1,339 protein groups. We could also show that the data obtained from FFPE tissues was in concordance with acetylation data from frozen tissues.

Conclusions: In summary, we present a reproducible sample preparation workflow optimized for FFPE tissues that resolves known proteomic-related challenges. We demonstrate compatibility of the S-trap with TMT labeling, offering high-throughput analysis. And, for the first time, we proved that it is biologically feasible to study endogenous lysine acetylation in FFPE tissues, contributing to better utility of the existing FFPE global archives.

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UVPD Fragmentation of Intact Proteins: Comparison of 193 nm versus 213 nm Photoactivation

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Topic: New Technologies – Mass Spectrometry

Introduction: Identification and characterization of intact proteins by top-down analysis depends on the depth of sequence coverage obtained during MS/MS events. The wide array of backbone cleavages produced by high-energy UVPD fragmentation, along with retention of post-translational modifications has proven useful for differentiation of proteoforms. The recent addition of a 213 nm laser on a commercial platform has enabled more widespread adoption of UVPD, which has motivated interest in understanding the differences between 193 nm and 213 nm fragmentation for top-down proteomics.

Methods: Various charge states for several model proteins introduced by heated electrospray ionization were isolated and activated using 193 and 213 nm UVPD using an OrbitrapTM Fusion Lumos system, optimizing activation time, AGC target, and averaging for maximum sequence coverage by Prosight database search. Additionally, number of laser pulses was varied for both UVPD wavelengths, and laser energy was varied for 193 nm UVPD. Custom R code was written to collate and analyze identified ions.
Results: The frequency of each ion type, location in the sequence, total sequence coverage, and preferential cleavages were investigated. For all proteins, a/a+1 and x/x+1 ions were identified most frequently for both 193 and 213 nm UVPD. In accordance with previous top-down 193 nm UVPD studies, performance of 213 nm UVPD had little dependence on the charge state analyzed, however decreased spectral congestion observed during UVPD of lower protein charge states appeared to aid both UVPD wavelengths. Average identified ion size tended to decrease as the number of laser pulses increased, indicating changes in the extent of secondary fragmentation. Heatmaps illustrating preferential cleavages by residue identity revealed enhanced fragmentation at phenylalanine and proline residues for both UVPD wavelengths.

Conclusions: Direct comparison of ultraviolet photodissociation for analysis of intact proteins using 213 and 193 nm photons revealed similar performance in all performance metrics.

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High resolution acquisition on a TIMS-QTOF for multiplexed targeted proteomics

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Topic: New Technologies – Mass Spectrometry

Introduction

prm-PASEF is a new targeted acquisition method that fully exploits the multiplexing capability and the high resolution of the TIMS-TOF mass spectrometer. Multiple peptides can be sequentially measured from a single ion mobility scan without compromising the sensitivity. We evaluated parameters including sensitivity, reproducibility, accuracy and dynamic range using AQUA peptides spiked in a Hela cell lysate. Finally, we applied the method to quantify the mutations and isoforms of the Ras oncoproteins family in cancer cell lines.

Methods
The quantitative performance of prm-PASEF was evaluated with a tryptic digest of HeLa cells spiked with 201 AQUA peptides and 15 light peptides. Serial dilutions ranging from 5.5 to 50,000 amole/µl were generated with 15 heavy/light peptides pairs. The other 186 AQUA peptides were spiked at the constant concentration of 2 fmole/µl. Ten cancer cell lines were analyzed for screening the Ras mutations. All acquisition were performed on a timsTOF Pro instrument.

Results :

The Aqua peptides calibration curves showed a signal response fitted by a linear regression over a concentration factor of 2900 (from 17.2 to 50,000 amole injected column), and an averaged RSD of 3% for the heavy/light peptide signal ratios. For label free quantification, the 2 fmole 186 AQUA peptides measured over 30 LC-MS runs showed a median RSD of 10.5% and a median of 25 data points across the chromatographic peaks. Finally, the G12 mutations of the Ras protein family as well as the three isoforms (NRas, KRas and HRas) were identified and quantified in 10 colon and lung cancer cell lines using a 10 min chromatography separation.

Conclusions

We developed a new targeted acquisition method that takes advantages of trapped ion mobility and fast high-resolution Q-TOF. The method is well suited for clinical applications that require to measure high density of targets with fast chromatography separations.

P146

DIA Performance in Discovery and Quantitation Analysis on New Orbitrap Exploris Mass Spectrometers

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Topic: New Technologies – Mass Spectrometry

Introduction
Data-independent acquisition (DIA) mass spectrometry (MS) provides deep proteome analysis without the bias from peak intensity. In addition to a more comprehensive coverage of identification, DIA also shows accurate label-free quantification. In this work, we evaluated the DIA performance on both Thermo ScientificTM Orbitrap ExplorisTM 480 and 240 mass spectrometers for discovery and quantitative proteomics analysis.

Methods
Mixed Hela:E.coli digested peptides (1:2, 1:4, 1:8 ratio) were loaded on 25cm Aurora column (25cm x 75um ID, 1.6um C18) with an Thermo ScientificTM Easy-nLCTM 1200 system, separated by a 90min LC gradient before being injected to Thermo ScientificTM Orbitrap ExplorisTM 240 or 480 MS. Mixed Hela:Yeast peptides was analyzed using the same setup at different gradient length (15min, 30min, 90min). Chromatogram spectral library was built by matching the gas-phase fractionations (GPF) to the predicted spectral library1. Acquired DIA data were analyzed by SpectronautTM 14.0.

Results
Spectral libraries of 1000ng Hela:E.coli mixture were built on two Orbitrap ExplorisTM platforms and the dynamic ranges were demonstrated to be up to 7 orders of magnitude. By matching with each library, 9056 and 9472 proteins were identified on Orbitrap ExplorisTM 240 and 480, respectively. The Hela:E.coli spike-in proteome acquired on both systems were quantified by directDIATM. ExplorisTM 480 exhibited better quantification accuracy than ExplorisTM 240. Furthermore, we also proved that matching with the library built at a longer gradient and a higher sample load would improve the identification coverage. Therefore, once built at ultimate setup, the spectral library can be used for universal DIA discovery of the same sample.

Conclusions
Both Thermo ScientificTM Orbitrap ExplorisTM 240 and 480 mass spectrometers had excellent performance in DIA analysis in discovery and quantitation.
References
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Robust Label Free Proteomics Performance with New Orbitrap Exploris 240 with Improved Upfront Separations

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Topic: New Technologies – Mass Spectrometry

Introduction
LC-MS-based proteomics analysis is a powerful analytical tool for identification and quantification of thousands of proteins in complex biological samples, but a LC/MS systems need to be robust and easy to use for large scale proteomics analysis without compromising on performance. Here we present a label-free proteomics workflow on a new quadrupole-Orbitrap hybrid mass spectrometer coupled to a High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) interface with a robust analytical setup without compromising on performance. The performance of this new benchtop mass spectrometer was evaluated in a Data-Dependent Acquisition (DDA) across different laboratories.

Methods
LC-MS analysis was performed with an EASY-nLC™ 1200 system with 200 cm, C18 25cm Aurora column (25cm x 75um ID, 1.6um C18) coupled to a new quadrupole-Orbitrap MS with FAIMS Pro Interface. Different concentrations of Pierce HeLa digest are analyzed in DDA, top speed mode with multi CV FAIMS peptide fractionation. Instrument baseline performance across different labs was evaluated using a 2µm, 15 cm PepMap C18 columns at 200 ng HeLa digest in a 60min gradient, DDA method without FAIMS. Proteome Discoverer™ 2.4 software with an improved peptide identification workflow was used data analysis providing improved peptide and protein coverage with a 1% FDR rate.

Preliminary data
Label-free proteomics performance was evaluated in different labs and resulted in ~5000 protein groups with ~10% additional improvement to protein coverage with FAIMS. Untargeted label-free quantitation of peptides in different ratio and with mixed proteome showed great quantitation accuracy. In summary the new Orbitrap Exploris 240 mass spectrometer has shown great sensitivity and data reproducibility for variety of proteomics applications in a multidisciplinary proteomics laboratory with ease of use for different level of user's expertise.
Novel aspect

A novel easy-to-use and robust Orbitrap mass spectrometer with optimized LC-MS and data analysis workflow for high throughput proteomics analysis.

P148

Benefits of Collisional Cross Section Assisted Precursor Selection (caps-PASEF) for Cross-linking Mass Spectrometry (XL-MS)

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Topic: New Technologies – Mass Spectrometry

Introduction – XL-MS has become an effective approach to uncover protein structural information. It utilizes small reagents that convert close proximity in protein structures into a covalent bond. Three products are obtained: linear peptides not captured by the reagent, mono-linked peptides captured by one side of the reagent, cross-linked peptides captured by the reagent within either the same protein or two different proteins. The desirable cross-links however consist of a mere 0.1% in a background of linear peptides making them difficult to detect. This is partially resolved with enrichable reagents exhibiting enrichment efficiencies of cross- and mono-links of >90%. This leaves the mono-links, for which we investigate the utility of trapped ion mobility separation to reduce this background and achieve more analytical depth on the timsTOF Pro.

Methods – Proteins were incubated with PhoX at 2 mM for 45 min. After reduction, alkylation and digestion with Trypsin, the mono-linked and cross-linked peptides were enriched from the background of linear peptides with Fe(III)-NTA cartridges on the Agilent BRAVO. Eluted mono- and cross-linked peptides were separated by nanoUHPLC over 70 min. Data acquisition on the timsTOF Pro was performed in PASEF mode.

Results –The timsTOF Pro efficiently detects cross-links at the extremely high speeds of operation of the PASEF mode. In addition, mono- and cross-links are gas-phase separated by the TIMS, helping to gain both analytical depth as well as uncover isotope patterns normally lost in the background. By converting the normal presentation of 1/k0 vs m/z to CCS vs mono-isotopic mass we find a clear separation between the two classes enabling a priori targeted precursor selection. This prevents ~60% of the mono-links, leading to a boost of ~20% in cross-links.

Conclusions – The timsTOF Pro represents an attractive option for increasing the analytical depth of cross-linking mass spectrometry.

P149

An Internal Standard-Triggered Parallel Reaction Monitoring Quantitative Assay for the Determination of Autophagy Activation

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Topic: New Technologies – Mass Spectrometry

Autophagy is an intracellular catabolic process that promotes the recycling of organelles and cytoplasmic components, acting as a regulator of homeostasis and cellular metabolism. Proteins involved in autophagy pathway remain challenging to measure in human muscles, due to their low abundance. The gold standard method to determine autophagy induction consists in measuring the lipidated form of LC3 and p62 proteins with western blotting. This methodology presents some limitations, in terms of sample throughput or multiplexing. Parallel reaction monitoring (PRM) has become an established approach to cope with such technological constraints, by improving sample throughput and providing precise quantification. A PRM method was developed for the assessment of 13 proteins putatively involved in autophagy in human muscles. Three proteins, including p62[1], were successfully quantified.

In order to increase the number of protein targets efficiently quantified, we implemented a so-called SureQuantTM internal standard (IS)-triggered quantification approach. SureQuant uses isotopically labeled IS as triggers to acquire, with high sensitivity, endogenous peptide signal in real time. One advantage of this methodology compared to classical PRM using time-scheduled acquisition, is that retention times of analytes are not pre-required. The mass spectrometer uses the detection of the IS peptide signals to trigger the measurement of their endogenous counterparts. When applied to the measurement of our set of proteins related to autophagy, SureQuant acquisition allowed the quantification of five proteins in total. For peptides quantified with both SureQuant and classical PRM, coefficients of variation for injection replicates were slightly better for SureQuant (i.e., 5% on average) than for PRM (i.e., 7.5%). Overall, SureQuant technique provides high quantitative accuracy, precision and specificity with increased analyte multiplexing capabilities.

[1] Poster at SMAP 2019 - Development of a Parallel Reaction Monitoring Assay for Quantification of Autophagy, Charlotte Macron, Gabriele Civiletto, Philipp Gut, Jérôme Feige, and Loïc Dayon.

P150

Removal Of PEG Contaminants From Peptides Through An Off-Line Ion-Exchange Spin Column

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Topic: New Technologies – Mass Spectrometry

Introduction
Consistent and reproducible sample preparation techniques are key to optimal mass spectrometric data acquisition. Contaminating substances such as polyethylene glycol (PEG) from plastic sources can obscure sample peaks in LC-MS analysis. The ability to perform sample clean up before loading the sample for analysis can ease bottlenecks and prevent column fouling. Here, we present a method for off-line ion-exchange clean up of samples using a modification of the ProTrap XG. The ProTrap XG is a two-stage filtration cartridge that facilitates sample precipitation and detergent removal. The filtration cartridge offers up to 99.8% purity and 95% recovery through acetone precipitation. An optional solid-phase extraction cartridge further cleans up the sample.

Methods
The spin-on reversed-phase chromatography cartridge from the ProTrap XG was replaced with a well-characterized, commercial strong cation exchange media. This proof of concept analysis used trypsin digested bovine serum albumin (BSA). The resin was primed, and the peptides in 15% acetonitrile (ACN), 0.1% trifluoroacetic acid were loaded by centrifugation. After a wash step, the peptides were eluted with 50 mM ammonium acetate pH 10, 100 mM KCl, 5% ACN and subjected to LC-MS. To prove the utility of the ion exchange column in sample clean up, samples were purposely spiked with PEG 400 then loaded onto the ion exchange cartridge, eluted and compared via LC-MS analysis to the same sample with and without PEG contamination.

Results
The use of the IEX cartridge resulted in a minimum of 90% recovery of the sample loaded as analyzed by LC-UV. Analysis of the samples by LC-MS showed no statistical difference in samples with and without ion-exchange treatment.

Conclusions
The use of an off-line ion-exchange chromatography step before mass spectrometric analysis results in a cleaner sample. This modification of the ProTrap XG can simply and reliably remove PEG from a processed sample.

P151

Improved immuno-affinity enrichment method for ubiquitinated peptides with high sensitivity, specificity and robustness

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Topic: New Technologies – Mass Spectrometry

Introduction
Ubiquitination is a critical post-translational modification in eukaryotic cells, triggering protein degradation or cell signaling events. A widely used method for ubiquitination site detection involves tryptic cleavage of ubiquitinated proteins generating a di-glycine remnant (K-GG) at sites of ubiquitination, followed by immuno-affinity enrichment using an anti-K-GG antibody and LC-MS/MS analysis. This method allows identification and quantification of thousands of sites of ubiquitination from cells and tissues but requires large starting sample amounts and results in co-isolation of many non-specifically binding unmodified peptides. In this study, we introduce an improved immuno-affinity enrichment method for K-GG peptides that overcomes these issues.

Methods
Tryptic mouse liver peptides were used to compare different types of beads, binding and washing buffers, and antibody amounts. Enriched K-GG peptides were analyzed on Thermo Scientific™ Q Exactive™ or Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometers and identified by SEQUEST software. A BCA assay was used to assess intact antibody elution.

Results
We have developed an optimized immuno-affinity enrichment method that provides 2-fold improved sensitivity, 2-3 fold improved specificity, and ~99% reduction in antibody released from beads upon acid elution. One milligram of mouse liver protein yields nearly 6,000 ubiquitinated peptides identified, numbers typically generated with 10+ mg of starting material using the previous method. The decrease in antibody eluted from the beads with K-GG peptides reduces the demand for C18 stage-tip cleaning and increases stability of the LC system. In addition, the optimized method uses magnetic beads instead of agarose beads, which considerably speeds and simplifies the procedure.

Conclusions
Optimized K-GG peptide immuno-affinity enrichment method significantly improves sensitivity, specificity, and robustness of ubiquitinated peptide identification and quantification.

P152

Microflow-Nanospray ESI-MS (MnESIMS) Platform for Human Plasma Proteomics

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Topic: New Technologies – Mass Spectrometry

Introduction: The Newomics award-winning multinozzle emitters (M3 emitters) enable optimization of sensitivity, throughput, and robustness by splitting microflow eluent evenly into multiple nanoflows, thereby dramatically enhancing ionization efficiency. We demonstrated the applications of our MnESI platform (microflow LC-nanospray ESI-MS) for both bottom-up and targeted proteomics studies of human plasma samples.

Methods: A 300-µm ID or 150-µm ID C18 column was paired with the Newomics MnESI ion source with M3 emitters to perform LC-MS analysis of human plasma digest on a Thermo QE-Plus and TSQ Quantiva MS. Sensitivity and robustness of MnESI platform at 1.5-µL/min were compared to nanoflow LC-MS at 0.3-µL/min using a Nanoflex ion source. For targeted proteomics analysis, performance of MnESI platform at 5-µL/min was compared to high-flow LC-MS at 250-µL/min using a HESI source.

Results: MnESI source with M3 emitter achieved an average sensitivity gain of 3.8 in peak area intensity relative to a single nozzle emitter at 1.5-µL/min. In addition, MnESI platform achieved the same sensitivity as nanoflow LC-MS at 0.3-µL/min. The CVs of peak area intensity and retention time from over 25 consecutive injections from MnESI platform were 50% smaller than that of nanoflow LC-MS.
For targeted proteomics, the average sensitivity enhancement from MnESI platform at 5-µL/min was about 50-fold compared to high-flow LC-MS. From 300 consecutive injections of plasma digests, an average peak area intensity CV of 4.8% for MnESI platform was achieved, compared to 6% on average obtained for high-flow LC-MS.

Conclusions: Newomics MnESI platform enables microflow LC-nanospray ESI-MS to deliver the same sensitivity as nanoflow LC-MS for bottom-up proteomics, while achieving better stability of retention time and peak area. MnESI platform achieves the same level of throughput and robustness as high-flow LC-MS for targeted proteomics, while dramatically increasing the sensitivity. MnESI platform opens new opportunities for clinical applications of human plasma proteomics.

P153

SureQuant targeted MS workflow for Quantitative analysis of Ras and AKT signaling pathways

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Topic: New Technologies – Mass Spectrometry

Introduction
The RAS/MAPK and AKT/mTOR pathways represent key mechanisms for cells to regulate cell survival, proliferation, and motility. The cross-talk between two pathways plays a central role in tumor progression and anti-cancer drug resistance. The quantitation of pathway protein expression and modifications are critical for characterization of disease, monitoring cancer progression and determining treatment response. A major limitation in the quantitation of pathway proteins is the lack of rigorously validated methods/reagents and a reliance on semi-quantitative results from Western blotting. We have utilized a novel SureQuant internal standard (IS)-triggered method applying a pool of reference internal standards to quantitate abundance of pathway proteins in a single MS run.

Methods
Multiple cancer cell lines (HeLa, A549, HCT116) were treated and processed using either EasyPepTM MS sample prep kits. 100 fmol of a combined mixture of 96 peptides for 46 pathway targets was spiked into 1 µg of digests. Samples were subjected to LC-MS analysis using Thermo Scientific Dionex nanoLC™ systems coupled to an Orbitrap Eclipse Tribrid Mass Spectrometer for SureQuant and Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer for PRM analysis. Data analysis was performed with Proteome Discoverer and Skyline software.

Results
In this study, we have developed a complete workflow solution by combining EasyPep MS sample prep kits and SureQuant targeted MS assays to quantitate biologically relevant signaling pathway proteins. The internal standards were spiked into digests from multiple treated cancer cell lines. Two targeted MS methods (PRM and SureQuant) were compared to assess the relative performance differences for quantitation of the desired endogenous peptides. More than 90% of the peptides were quantitated using the SureQuant analysis from multiple cancer cell lines.

Conclusions
SureQuant targeted MS analysis allows reproducible, routine and simultaneous quantitation of functionally relevant targeted peptides from signaling pathway proteins.

P154

Quantification of Common and Troublesome CHO-HCPs by Internal Standard-Triggered Assay of Heavy Tryptic Peptides

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Topic: New Technologies – Mass Spectrometry

Introduction

Quantifying host cell proteins (HCPs) during process development of biopharmaceuticals is critical in understanding and optimizing conditions for their removal. Mass spectrometry-based methods can offer high specificity and accurate determination of HCP concentrations making it a suitable alternative to ELISA based techniques. Herein, we developed methods to trigger endogenous HCP peptide measurements by MS detection with spiked-in heavy labeled versions of corresponding target peptides. The heavy stable isotopically labeled peptides function as a quantitative reference standards as well as a marker to dynamically guide MS monitoring.

Methods

Clarified harvests and several in process samples of both a vaccine molecule and monoclonal antibody expressed in Chinese Hamster Ovary cell lines (CHO) were digested with Trypsin using standard protocols. A standard curve was generated by spiking in a series of heavy-labelled concentrations. LC-MS was performed using a Duo UHPLC Vanquish coupled to a Thermo Scientific Fusion Lumos MS using a custom SureQuant internal standard-triggered quantitation approach. Results were analyzed by Skyline and Proteome Discoverer.

Results

Preliminary DDA based HCP data and Proteome Discoverer searches yielded a list of the most common host cell proteins expressed in CHO with a dominant focus on proteases or lipases that may adversely affect product quality. Accordingly, a new SureQuant peptide panel was developed to provide accurate, absolute quantitation of 282 peptides in a single run. This assay was implemented to analyze a series of process samples in two separate samples, a vaccine molecule and a multi-specific antibody. Lastly, a new “winged” peptide approach was used to correct for sample handling losses ultimately enhancing the accuracy of this approach.

Conclusions

HCP analysis using the novel SureQuant panel provides an accurate approach for HCP quantitation while offering high dynamic range ultimately providing a new and reliable approach for routine HCP analysis and quantitation.

P155

Peptide retention time prediction for TMT-labeled peptides in 2D LC-MS/MS experiments

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Topic: New Technologies – Mass Spectrometry

Introduction

Quantitative proteomic studies rely heavily on the application of various stable isotopes labeling techniques such as tandem mass tags (TMT). In this work we attempt to understand the effect of TMT labeling on chromatographic behavior of tryptic peptides in various peptide separation modes applied in proteomics. Having the ability to predict retention properties of modified peptides will streamline method development for quantitative proteomics and improve confidence of peptide identification for TMT-based quantitative studies.

Methods

Our approach is based on retention data collection for non-modified and TMT-labeled peptides from tryptic digests of human cell lysates (A546, HCT116, MCF7, Jurkat) using 2D LC-MS/MS analyses (Thermo Scientific™ Q Exactive™ HF-X platform with a Thermo Scientific™ EASY-Spray™ 50 cm x 75 µm column). 4 different separation modes have been used in the first dimension: HILIC-amide, HILIC-silica, SCX and high pH RP. Retention values for identified peptides (HI, hydrophobicity index; % acetonitrile) were accurately measured using spiked standard peptide mixtures. Retention shifts upon TMT modification expressed HI units or fraction number have used to elucidate the effect of modification on peptide retention properties in the second and first dimensions, respectively.

Results

Combined collections of ~145,000 and ~15,000 unique non-modified/TMT labeled peptide pairs were used for retention modeling in the second (reversed-phase, formic acid) and four different first dimension separations, respectively. TMT modification increases peptide retention by 2.7% acetonitrile, 8.6% acetonitrile, 6.5% water and 0.6% water on average in low pH RP, high pH RP, HILIC-silica and HILIC-amide separation modes, respectively. SCX retention remains virtually the same.

Conclusions

A number of composition and sequence-specific effects drives alteration of peptides' chromatographic properties following modification with TMT. These features have been applied for the development of respective Sequence-Specific Retention Calculator (SSRCalc) -TMT peptide retention prediction models, covering various peptide separation modes.

P156

Millisecond informatics: next generation real-time analytics for quantitative proteomics

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Topic: New Technologies – Mass Spectrometry

Recent developments in intelligent data acquisition strategies are leading the next generation of multiplexed proteome quantitation. Real-time peptide spectral matching enables superior quantitation and speed compared to canonical methods (Schweppe, JPR 2020). Intelligent data acquisition strategies require millisecond time-scale informatics pipelines for real time decision making. These included spectral pre-processing, analysis, peptide spectral matching, and false discovery rate estimation for spectral filtering. In this work we present a first-of-its-kind, fast and robust informatics workflow that is capable of assigning peptide-level false discovery rates on a millisecond time scale with high fidelity to offline statistical methods. The millisecond informatics pipeline, Orbiter, was built in C# to support interfacing with the Thermo Fusion API for online instrument control. Orbiter combines fast data acquisition with accurate quantitation of high dynamic range changes. The former of which was canonically accomplished by HRMS2 methods; the latter of which was canonically accomplished with SPS-MS3 methods. The Orbiter pipeline employs real-time peptide spectral matches (PSMs) for selective SPS-MS3 quantitation only when a “good” peptide is identified. Orbiter consists of three central modules: (1) preprocessing, (2) searching, and (3) filtering. Spectral preprocessing facilitates monoisotopic peak estimation to improve peptide identification rates. In the second module, real-time searching with Comet determines PSMs in parallel with instrument acquisition. In the filtering module, SPS ions are selected based on relative spectral purity and peptide termini. Simultaneously, the false discovery for each new PSM is estimated based on linear discriminant analysis. Stochastic local FDR fluctuation are controlled by monotonic regression. The final level of filtering encompasses inclusion and exclusion lists at both the peptide and protein level for user-assigned restriction of SPS-MS3 quantitation. The result is a highly flexible, robust platform that can be employed on a millisecond timescale necessary for incorporation with IDA strategies for real-time, adaptive data acquisition.

P157

Toward a Single Comprehensive Assay for Protein Expression of the Human Kinome

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Topic: New Technologies – Mass Spectrometry

Introduction

The human kinome is comprised of 518 protein and lipid kinases which play a fundamental role in the propagation and integration of cell signaling events. Dysregulation of kinase expression and activity plays a critical role in human disease with >55 kinase inhibitors in the clinic or late-stage trials. Inhibitor resistance develops, in part, due to an adaptive response involving multiple classes of kinases. Yet, comprehensive quantification of cellular kinase levels remains challenging. We report a single LC-MS assay that reproducibly quantifies 976 stable isotope labeled peptides for 367 kinases in a single LC-MS analysis using a new internal standard-triggered acquisition method.

Methods

976 isotope labeled peptide standards corresponding to 367 human kinases were separated on an Easy nLC 1200 pump with a PepMap C18 column (75µm x 15cm) and 90min gradient at 300nL/min. MS analysis was performed on an Orbitrap Exploris 480 instrument. Standard peptides in solvent were analyzed for creation of a spectral library and construction of a custom SureQuant instrument method. The SureQuant assay was then applied to quantification of kinase expression in 1.5µg SUM159 whole cell tryptic digest with 150 fmol internal standard peptides.

Results

Using an optimized SureQuant method, we identified 960 of the 976 internal standard peptides from 367 kinases from a single 90 min LC-MS analysis. The LC-MS quantification of kinases usually requires an enrichment step. Using the SureQuant method, we could quantify 650 peptides from 317 kinases in un-enriched, single shot LC-MS analysis of tryptic digests of whole cell lysates. This is in contrast to scheduled PRM-based quantitation using 8-minute windows, which requires 3 2hr LC-MS analyses and yields quantitation for fewer than 90 kinases.

Conclusions

The SureQuant method with stable isotope labeled standards shows promise as a high-throughput high-precision strategy for comprehensive kinome quantification using enriched and un-enriched complex biological samples.

P158

Capillary chromatography for intact therapeutic proteins and their subunit analyses using MAbPac RP column

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Topic: New Technologies – Mass Spectrometry

INTRODUCTION: The state of art, mass spectrometry based analyses of therapeutic proteins and their subunits has become an important approach in biopharmaceutical industry. Post translational modifications such as glycosylation, glycation, C-terminal lysine truncation, deamidation, etc. can easily be analysed at subunit level. A new capillary column has been developed to perform these important characterizations with low flow chromatography. The improved sensitivity offered by capillary flow is especially important in intact protein analyses due to the improved ionization efficiency.

METHODS:Intact mAbs were buffer exchanged using 10k cut off centrifugal filters. For intact analyses, Fc glycans of mAbs were digested with EndoS to assess high mannose and ratio of fucosylated/afucosylated glycoforms. To generate heavy and light chains, mAbs were reduced with 15 mM TCEP, while further breaking the mAbs down into Fc, LC and Fd' subunits required IdeS digestions followed by reduction and clean up. Intact proteins were analysed in low resolution mode (17,500), subunits and glycopeptide hormones were analysed at 140,000 resolution in intact protein mode using a hydrid Orbitrap mass spectrometer.

RESULTS: Characterization of intact proteins in capillary flow showed 140 times more sensitivity in ESI without compromising data quality. As noticed, in the case of Trastuzumab, the originator has higher level of galactosylation than the biosimilar. The normal mode (low resolution) or HMR mode are only applicable for mAbs with Fc glycosylation. Subunit analyses is the only way to characterize one of the mAbs of this report with FAb glycosylation. Subunit analyses in intact protein mode isotopically resolves the subunits and shows the presence of sialylated, hybrid and surprisingly high amount of Man5 located on Fd'. In addition, the new column separates very well tryptic peptides as well.

CONCLUSIONS: High resolution, sensitive characterization of intact proteins and their subunits in capillary flow.

P159

A Versatile Isobaric Tag Enables Proteome Quantification in Data Dependent and Data Independent Acquisition Mode

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Topic: New Technologies – Mass Spectrometry

Introduction: Quantifying proteins based on peptide-coupled reporter-ions is a multiplexed quantitative strategy in proteomics that significantly alleviates the problem of ratio distortion caused by peptide co-fragmentation, as commonly observed in other reporter-ion based approaches, such as TMT and iTRAQ. Fueled by improvements in mass spectrometry and data processing, data-independent acquisition (DIA) is an attractive alternative to data-dependent acquisition (DDA) due to its better reproducibility. While multiplexed labeling is widely used in DDA, it is rarely used in DIA, presumably because current approaches lead to more complex MS2 spectra or to a reduction in quantification accuracy and precision. Herein, we present a versatile acetyl-alanine-glycine (Ac-AG) tag which conceals quantitative information in isobarically labeled peptides and reveals it upon tandem MS in the form of peptide-coupled reporter-ions. Since the peptide-coupled reporter-ion is precursor-specific, while fragment ions of the peptide backbone originating from different labeling channels are the same, the Ac-AG tag is compatible with both the DDA and the DIA mode. By isolating the monoisotopic peak of the precursor ion in DDA, intensities of the peptide-coupled reporter-ions simply represent the relative ratios between constituent samples, while in DIA the ratio is inferred after deconvoluting peptide-coupled reporter-ions.

Method: The proteome quantification capability of the Ac-AG tag was demonstrated by triplex labeling of a yeast proteome spiked with bovine serum albumin (BSA) over a 10-fold dynamic range.

Result: Within a complex proteomics background, the BSA spiked at 1:5:10 ratios was detected at ratios of 1.00 : 4.87 : 10.13 in DDA and 1.16 : 5.20 : 9.64 in DIA.

Conclusion: We describe an isobaric labeling approach based on the Ac-AG tag that maintains the advantages of existing peptide-coupled reporter-ion based quantification methods in DDA but also allows multiplexing in the DIA mode without sacrificing the rate of data acquisition or complicating MS2 spectra.

P160

MRM-based quantification of plasma Apolipoprotein A-I and B100 to help in identifying coronary-artery disease

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Topic: New Technologies – Mass Spectrometry

Background: Apolipoprotein A-I(ApoA-I) constitutes the major component of high-density lipoprotein(HDL) and Apolipoprotein B-100(ApoB-100) is the major protein constituent of low-density lipoprotein(LDL) and also constituents about 40 percent of the protein moiety of very low density lipoprotein(VLDL) and chylomicrons.The Apo B/ApoA-I ratio is a strong predictor of cardiovascular disease risk.Traditionally,ApoA-I and Apo B-100 are quantified using immunoassays,which may suffer from problems such as cross-reactivity.A quantitative LC-MS/MS method for specific and simultaneous measurement of Apo A-I and B-100 has been developed for assessment of cardiovascular risk.

Methods: Apolipoprotein calibrators from Randox Laboratories were used for external calibration with Apo A-I and B100 concentrations traceable to WHO international reference materials.The tryptic digested peptides were analyzed by LC-MS/MS. The quantifying peptide ATEHLSTLSEK for ApoA-I and FPEVDVLTK for ApoB-100 was selected based on CPTAC assay portal. Synthetic Isotope labeled peptides (SIS) were used as internal standard. The LC-MS/MS results were compared to those from Nephelometric assay.

Results: Our assay showed a linear range of 0.12-2.41g/L for ApoA-I and 0.12-2.31 g/L for ApoB-100 with R-square greater than 0.99.The LOQ is 0.12 g/L of ApoA-I and ApoB-100 in plasma.The Intra- and inter-assay coefficients of variation were less than 10%. The LC-MS/MS results of plasma ApoA-I and ApoB-100 correlated well with those from the existing Nephelometric method (Beckman IMMAGE). The Passing&Bablok(95%CI) slope is 1.03 for ApoA-I and 0.98 for ApoB-100 for 60 plasma samples.The Pearson correlation coefficient was r=0.97 and 0.98 for ApoA-I and ApoB-100.24 external quality control samples from CAP EQA program were analyzed ApoA-I and ApoB-100 level and the measured values were within +/-2.0 z-score from the peer mean.

Conclusions: A LC-MS/MS method was developed for the accurate quantitation of ApoA-I and B100 aiding to identify cardiovascular disease.1.Irene VD Broek,Fred P.H.T.M. Romijn,and Christa M.Cobbaert, et al, Clin Chem,2016,62,188-197;2.Michel R. Langlois, et al, Clin Chem,2018,64,1006-1033;3.Michael E. Lassman, et al, Rapid Commun.Mass Spectrom.2012,26,101–108

P161

Deep learning enables automated and extensible peak group identification for multi-transition chromatogram-based data-independent acquisition data analysis

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Topic: New Technologies – Mass Spectrometry

Introduction
Data-independent acquisition (DIA) is a novel mass spectrometric method that achieves high reproducibility and quantitative accuracy through a deterministic acquisition strategy; however, existing heuristic and knowledge based software cannot keep up with the increasing complexity of the resulting data. Here, we present a novel method based on deep learning (DL), which is able to automatically extract and classify peak group features directly from chromatographic DIA data. Our approach is an end-to-end neural network that performs feature extraction and scoring in one single step, unlike previous approaches that relied on manual feature engineering. Our method is capable of capturing more information from the raw data and is easily modifiable to be compatible with additional sources of information.

Methods
We used a S. pyogenes dataset of 304,000 chromatograms from 16 equal sized LC-MS/MS runs, of which 7,232 were manually annotated with exact peak group information (452 peptides). Target and decoy chromatograms were extracted with OpenSWATH to generate multi-transition chromatographic data as well as library information from the raw data and assay library files and converted into NumPy arrays for use as inputs. Each separate group (unlabeled, manually validated, and decoy) of chromatograms was randomly split into 5 folds for cross validation. A deep neural network was trained on this data to identify peak groups, and compared against the results of an XGBoost based PyProphet model trained on the OpenSWATH identified peak groups.

Results
Deep learning model achieves comparable recall at low (0.01-0.05) false discover rate (FDR), and superior recall at higher FDR, compared to current non-deep learning state of the art. Qualitative results also suggest tighter peak boundaries, which may suggest the possibility of better quantification.

Conclusions
Very first DL model that directly analyzes XIC data from DIA/PRM data and outperforms existing algorithms in peak group identification.

P162

PASEF-PRM LIVE on the timsTOF Pro

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Topic: New Technologies – Mass Spectrometry

Introduction
Integrated online ion mobility coupled with targeted MS/MS affords an opportunity for rapid and highly selective detection of peptides in complex matrices. However, leveraging the full power of this approach requires reproducible LC retention times for all targeted peptides across numerous sample injections. This level of reproducibility can be difficult to achieve in practice, particularly with smaller ID columns and lower LC effluent flow rates often used to maximize ionization efficiency. Use of wider retention time windows can offset retention time drift, albeit at the cost of fewer peptides in each targeted analysis. Here we introduce PASEF-PRM LIVE, a framework that implements on-the-fly scheduling of MS/MS events to maximize target peptide coverage while responding to changes in target elution times.

Methods
We have developed a Python API that allows real-time control and execution of PASEF-PRM scans which we call PASEF-PRM LIVE. The method monitors elution of user-specified landmark peptides and then uses a regression model to predict the elution times of upcoming peptides on the target list. This allows the acquisition engine to tolerate changes in retention time and affords a higher density of peptides targeted for quantification.

Premilitary Results
We queried ~100 peptide targets in human biofluids to test the performance of several time-warp algorithms to dynamically accurately adjust precursor scheduling parameters. We found that more than 90% of peptides were correctly targeted for PRM even when the LC gradient was manually altered to mimic dramatic retention time shifts. Person correlation was more than 95% between replicate injections, indicating that our PASEF-PRM LIVE framework accurately tracked retention time drift and adjusted acquisition parameters. Representative data for targeted analysis in complex proteomes by LC-MS/MS and CE-MS/MS will be discussed.

Conclusions
Our framework for PASEF-PRM LIVE acquisition on the timsTOF Pro will dramatically increase the throughput of peptide PRM quantification.

P163

Development of a multiplexed protein panel using a targeted proteomics approach for the study of CDK4/6Inhibitors resistance in breast cancer

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Topic: New Technologies – Mass Spectrometry

Introduction: Recurrent and metastatic disease limit the survival of patients with hormone receptor-positive breast cancer. Despite the improved disease control with CDK4/6Inhibitors, not all patients respond to these therapies. Our aim is to perform a quantitative evaluation of marker proteins with a developed multiplexed panel using targeted-mass-spectrometry-based proteomics for 25 proteins central to CDK4/6Inhibitors resistance.

Methods: We developed Multiple Reaction Monitoring(MRM)-MS methods for the 25 target proteins using synthetic heavy-isotope-labeled standards with the aim of creating MRM assays to enable specific, sensitive and precise quantitation of these proteins in small amounts of samples. We developed a high-resolution peptide fractionation system using high-pH micro-flow liquid chromatography(LC) which is required to overcome the problem of small sample amounts while improving analytical assay sensitivity in the analysis of complex biological matrices such as biopsies.The MCF-7 human breast cancer cell line was used as model during method development. Proteins from cell lysates were digested with trypsin. The resulting peptides were micro-flow fractionated into 70 fractions and the developed nano-LC-MS-MRM assays were used for peptide detection and quantification.

Results: Our developed micro-flow fractionation method allowed us to work on limited amounts of samples (60ug) and increased the possibility to detecting low abundance proteins such as cell cycle components. Using the MCF-7 cell model, we are able to identify and quantify 17 proteins from our panel: Cdk1-2-4, CyclinB1-D1-D3-E1, RB1, E2F3-4-5, ESR1, TOP2A, TYMS, EZH2, MKI67, BIRC5.

Conclusions: We have developed a highly specific MS-based multiplexed assay with peptide standards targeting 25 proteins relevant to CDK4/6Inhibitors breast cancer treatment. Our micro-flow fractionation method increased assay sensitivity and allows for the analysis of small sample amounts. In the future we will apply this workflow to Patient-Derived Xenografts models, breast cancer tissues and FFPE samples to identify the predictive value of these potential biomarkers for responsiveness to CDK4/6Inhibitors.

P164

A Proteomic sample preparation for mass spectrometry using an automated workstation

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Topic: New Technologies – Non-Mass Spec

Mass spectrometry (MS) -based protein and peptide quantification is increasingly being applied as a bio-analytical tool. Sample preparation serves as the foundation for any proteomic analysis. We have developed an automated workstation workflow to perform all necessary plasma sample preparation procedures including desalting step. In our improved method, an automated workstation was programed to perform all necessary plasma sample preparation procedures: denaturation, reduction, alkylation, digestion and desalting. After processing, the samples were run on a triple quadrupole LC-MS in an SRM assay targeting serum albumin, -galactosidase. The coefficient of variation (CV) of SRM signal for each transition was used to monitor the reproducibility of automated digestion protocol. MRM signals from these five SIL peptides were acquired to monitor the precision of automated liquid transferring steps. The average %CV for peptides, DDNPNLPR^, and GDFQFNISR^ (^ represents the N15 labeled amino acid) from Reaction Mix 1 step ranged from 1.8% to 11.2%. The average %CV of one peptide (IDPNAWVER^) from the Cysteine Blocker step ranged from 6.6 to 8.8%. The average %CV of two peptides (WVGYGQDSR^ and LVNEVTEFAK^) ranged from 6.2% to 11.9%. Intra-day CVs were calculated from 21 wells prepared on the same day. The mean intra-day %CVs for 40 proteins ranged from 4 - 20%. To evaluate the edge effect of the plate based automated workflow, %CV was calculated from specific wells within designated columns and rows. MRM signals intensities were similar in all column and row configurations with %CV ranging from 3 - 22%. In summary, the optimized automated workflow yields 96 uniformly-processed samples in less than five hours with excellent experimental precision. The automated workflow presented here provides for consistent enzymatic digestion with improved reproducibility and throughput compared to manual methods.

P165

Phosphopeptide Recovery Enhancement in LC/MS Studies Using Columns Treated with a Novel Surface Barrier

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Topic: New Technologies – Non-Mass Spec

Despite the continued advances of mass spectrometry as an analytical technique, detection of phosphopeptides by liquid chromatography coupled with mass spectrometry (LC/MS) still remains a challenging application within proteomics. A major problem is that phosphopeptides do not protonate efficiently due to the presence of one or more acidic phosphate groups, making their detection difficult. However, there are other significant mechanisms which contribute to the difficulties in phosphopeptide analysis by LC/MS.

Incomplete recovery from the wetted components of an LC system is another major cause of poor phosphopeptide detection. Metal ions interact with the phosphate group via an ion-pairing mechanism, resulting in partial or even complete retention and poor peak shape. Generally, the more phosphate groups a peptide contains, the worse their recovery and chromatographic peak shape. Current strategies to improve phosphopeptide recovery in LC/MS analyses include the addition to the sample of EDTA or citrate, with both acting as metal chelators, or multiple injections of these solutions to attempt passivation of the fluidic path prior to sample analyses. However, these strategies do present drawbacks as they can cause issues with chromatographic performance and sensitivity of the MS measurement. An alternative approach is a new, highly effective surface barrier, comprising of a hybrid inorganic/organic silica surface which mitigates undesired interactions with metal surfaces. Phosphopeptides resulting from a variety of tryptically digested proteins were analysed to highlight the recovery benefits provided by the surface barrier technology. The recovery rates were shown to vary based upon the number of phosphorylation sites and number of amino acid residues within the peptide sequence, however, in some cases phosphopeptides not previously observed were detected with high signal intensities when acquired with the barrier technology. These new surfaces are shown to limit the metal ion interaction with the phosphate group and improve phosphopeptide recovery over current workflows.

P166

Simplified high-throughput methods for deep proteome analysis on the timsTOF Pro

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Topic: New Technologies – Non-Mass Spec

Introduction: Established proteomic methodologies rely on long analytical columns and extended UHPLC gradients to achieve comprehensive proteome coverage. The timsTOF Pro pushes fragmentation rates beyond 100Hz and provides accurate collisional cross section (CCS) with established methodologies underutilising these advances. High-throughput proteomics for large-scale protein characterization, screening and expression was previously limited by gradient length and instrument sensitivity. In this study, we have developed novel LC-MS methods for packed emitter columns that fully utilise instrument speed and CCS values to enable the analysis of high numbers of protein expression profiles in less than a day.

Methods: Digested HeLa and plasma were analysed to determine the sensitivity and robustness of the novel methods. Samples were fractionated using RP-HpH. Peptides were injected by UHPLC coupled online to a timsTOF Pro mass spectrometer. Peptide separation was performed using (15cm x 75µm i.d.) or (5cm x 150µm i.d.) 1.6 µm C18 packed emitter columns. A gradient was run for 17 min or 5 min, respectively. 4 dimensional (4D) peptide data was acquired using ddaPASEF before analysis using MaxQuant.

Results: We combined rapid generation of deep peptide libraries with enhanced matching of single shot ddaPASEF sample analysis with only a 17 minute separation gradient (50 samples per day). The combination of high-performance chromatography and CCS enhanced library-based matching resulted in an average of 6000 protein identifications within individual single shot samples from HeLa digests. Additionally, an ultra-high throughput setup utilizing 5 min gradients (180 samples per day) yielded >3600 protein identifications per sample. The use of 5 min gradients to analyse neat plasma resulted in the robust quantitation of >200 proteins per sample.

Conclusions: These 4D LC-MS ddaPASEF workflows are simple to implement on available technology and do not require complex software solutions or custom-made consumables to achieve high throughput, deep and robust proteome analysis.

P167

New Approaches for Quantification of SIL Peptides for Targeted Proteomics

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Topic: New Technologies – Non-Mass Spec

Introduction
Absolute protein quantification by targeted proteomics is dependent on the availability of absolutely quantified stable isotope labeled (SIL) peptide standards. To provide these fast and economically, a new method for absolute peptide quantification has been developed in which the peptide is quantified by HPLC-UV using an attached quantification tag with a specific UV absorption (1, 2). Here we report data on the validation of the method and on an extension of the approach to allow direct quantification of reconstituted UV-tagged peptides by the end-user.

Methods
Several model peptides were prepared at high purity (>95%) and absolutely quantified by the quantification tag approach using UV-HPLC measurement against an absolutely quantified UV-reference standard. The peptides were resuspended and quantified by different methods: a) measurement of specific UV absorption at different wavelengths using a UV photometer (Nanodrop), b) HPLC-MS measurement, and c) amino acid analysis (AAA).

Results
The developed quantification method was linear over a wide concentration range and the quantification tag could be efficiently removed by tryptic digestion. The comparison of different quantification methods, all applying the new quantification tag approach, revealed a very good correlation between the methods and with AAA. Details will be given on the used peptides, the choice of solvent, and the variation (CV) between the methods.

Conclusions
A new approach for efficient quantification of UV-tagged SIL peptide standards is described. The method has been extended to enable accurate and simple end-user based quantification of peptide solutions within targeted proteomics workflows.

(1) Schnatbaum, K. et al., Proteomics 2020, 20, 2000007.
(2) Recent applications: e.g. (a) Blacher, E. et al. Nature 2019, 572, 474-480. (b) Naggie, S. et al. Proteomics Clin. Appl. 2019, 13(3), e1800006.

P168

Assessment of streptavidin bead binding capacity to improve quality of streptavidin-based enrichment studies

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Topic: Other

Introduction: Streptavidin-based enrichment of biotinylated molecules is a common methodology used routinely across multiple disciplines in biomedical research. Numerous and varied formats of immobilized streptavidin are available, but predicting which product is most apt for a given application is complicated by the fact that there are no universal reporting standards for describing binding capacity of the beads.

Methods: We developed a colorimetric competitive displacement assay, the streptAVIdin binDing capacITY (AVIDITY) assay, for assessing the binding capacity of streptavidin beads. The assay is based on the competitive displacement of HABA from streptavidin by biotin resulting in a spectroscopic change due to HABA converting from bound to unbound state. To assess the ability of the AVIDITY assay to evaluate differences in binding capacity products from the same vendor and between different vendors, the assay was applied to streptavidin bead products from a variety of vendors. Cell Surface Capture (CSC), a discovery-driven mass spectrometry approach that selectively enriches biotinylated cells surface N-glycoproteins, was used as a downstream application to assess the results of the AVIDITY assay. MS data were acquired using an Orbitrap Exploris 480 and processed using Proteome Discoverer.

Results: Our results demonstrate that the AVIDITY assay is sensitive enough to detect differences in binding capacities of Invitrogen streptavidin Dynabeads products and allowed us to correlate these differences in binding capacity to N-glycosylated peptide identifications in CSC experiments. Our results show that the assay works well among beads that are different sizes and composed of different substrates.

Conclusions: We expect this assay to benefit users across disciplines to make informed decisions regarding the most apt streptavidin bead products for their own experiments.

P169

A targeted proteomics biomarker signature for the detection of malignant pleural mesothelioma from the blood

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Topic: Other

Introduction: Malignant pleural mesothelioma (MPM) is an aggressive thoracic cancer caused by the exposure to asbestos. Routine clinical blood biomarkers to detect the cancer early or to stratify patients on their prognosis are currently missing. In our work, we have verified a multivariate targeted proteomics signature for the detection of MPM from the blood (1). The original signature composed of seven N-linked glycopeptides was identified in previous work in cell lines and blood. A reduced version of the signature composed of six N-linked glycopeptides was investigated in the current work in cohorts of MPM patients and asbestos exposed donors.

Methods: We have applied selected reaction monitoring (SRM) targeted proteomics in serum samples from a multicenter cohort of more than 400 MPM patients and asbestos exposed donors. Samples were processed on 96-well plates to enrich for N-linked glycopeptides.

Results: The biomarkers composing the signature did not present apparent functional or physical relation, highlighting the unbiased approach of a proteomics strategy for biomarkers identification. The integration of the biomarkers into a multivariate signature increased the discriminatory accuracy compared of using a single biomarker at a time, highlighting the power of the multiplexed biomarkers strategy. The signature produced AUC (area under the receiver operating characteristic curve) of 0.738 for discriminating MPM and asbestos exposed donors. For early stage MPM patients (stage I/II) the AUC was 0.765 and the negative likely-hood ratio was 0.11, underlying the sensitivity of the multivariate biomarkers signature. Furthermore, the signature presented prognostic potential among the MPM patients.

Conclusion: The multivariate biomarkers strategy based on targeted proteomics has important potential for MPM diagnostics from the blood.

Reference: 1. Cerciello, F., et al. 2020, Cancer Epidemiol Biomarkers Prev. 2020 Jul 30:cebp.0543.2020

P170

Cross-lab evaluation of quality control for large-scale DIA-MS proteomics

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Topic: Other

Introduction: Quality control (QC) of LC-MS/MS platforms is critical for generating reproducible and high-quality proteomic data. Multiple QC procedures have been established based on data acquired in data-dependent acquisition (DDA) mode using standard samples. Data independent acquisition (DIA) MS-based proteomics has demonstrated high degree of reproducibility and throughput. However, there is no comprehensive QC procedure for large-scale DIA-MS.

Methods: The C57BL/6J mouse liver digests were analyzed twice a week and once before/after each instrument maintenance with both DDA and DIA mode in 19 MS instruments in nine laboratories from both academic and industry for up to 12 months. The MS instruments investigated in our study included Q-Exactive, QE-HF, QE-HFX, Exploris 480, TripleTOF 5600+ and 6600, timsTOFpro. Both nanoflow and microflow LC systems were included.

Results: We collected 800 DDA runs and 800 DIA runs of mouse liver digests from five MS instruments for about 12 months, and systematically compared the MS1 and MS2 signal intensity, number of spectral scans, peptides and proteins based on the DDA and DIA QC runs in good and various suboptimal conditions. Our data showed that while protein identifications from DDA remained same, the abundance of 128 manually inspected peptide precursors in DIA reduced by >50% in certain cases, suggesting that DIA-based QC is more sensitive than DDA-based QC.

Further refinement using statistics led to the identification of about 100 peptide precursors which demonstrated high degree of specificity for various conditions for poor data quality. Finally, we evaluated these mouse liver DIA-QC peptides in eight collaborative laboratories. More details on the study results will be discussed.

Conclusions: We investigated the feasibility of using DIA data for QC and established a protocol for DIA-based QC.

P171

Optimizing label-free proteomics in non-human primates

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Topic: Other

Introduction: The overall effectiveness of the label-free proteomics approach is not only dependent on the method of data acquisition in the mass spectrometer, but equally dependent on the downstream data processing, including software tools, query database, data normalization and imputation. Label-free proteomics becomes particularly challenging if the query database of a model organism is not comprehensively annotated as in the case of non-human primates. In this study we examined label free proteomics in non-human primates to maximize qualitative and quantitative data information.

Methods: Our study included 45 brain frontal cortex samples from baboons in the age range of 6-23 years (22-80 human equivalent years). Samples were homogenized, followed by reduction, alkylation and tryptic digestion of proteins. Resulting peptides were separated on a C18 easy spray column using a 2-hour gradient and analyzed on Fusion Lumos mass spectrometer.

Results: Raw data analysis using MetaMorpheus software identified 2852 proteins with 34 different post-translational modifications. Only 112 proteins were identified with no missing values in all 45 samples. To improve the quantitative data information and fill in the missing data points, we tested 20 different imputation methods on our dataset where values were manually removed and imputed back to check the imputation accuracy of each method. The top performing methods were tested further to assess the effect of missing data percentage on the imputation accuracy. Our analysis suggests that imputation methods taking overall data structure into consideration (random forest or k-Nearest neighbor) perform better in comparison to methods which replace the missing data with a constant minimum value or column median. Here, the imputation accuracy is close to 90% even if 50% of the data is missing.

Conclusion: Our study offers a detailed comparative analysis of various aspects of label free proteomics for maximizing data information in non-human primates.

P172

Characterization of Transglutaminase-Directed Chromophore-Tagged Proteins by Ultraviolet Photodissociation

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Topic: Other

Introduction: The ability to selectively tag proteins with chromophores has sweeping applications ranging from cellular imaging to mapping protein-protein interactions. The most common methods for labeling proteins typically target nucleophiles, e.g., primary amines or cysteines. These functional groups are abundant in protein sequences, therefore the tagging process is rather non-specific. Targeting alternative functional groups offers complementary selectivity, and the development of new protein engineering tools allows tagging of less common residues, such as glutamine, by using transglutaminases (TGase). UV chromophores are installed on the glutamines in a robust and site-selective manner, thus endowing only those peptides that contain glutamine to be responsive to UVPD using 355 nm photons.

Methods: Protein was directly conjugated by enzymatic-assisted derivatization using microbial transglutaminase (mTGase) in potassium phosphate to install the UV chromophore tag. Modified protein was subjected to tryptic digestion. Trypsin (1:20 w/w) was added to the solution and incubated at 37 °C for 4 h. Samples were desalted with Pierce C18 spin columns and resuspended for LC conditions. Analysis was performed on a Thermo Scientific Velos Pro dual-pressure linear ion trap mass spectrometer equipped with a Dionex UltiMate 3000 liquid chromatograph and a Continuum Nd:YAG laser tuned to 355 nm.

Results: A model peptide with a single glutamine underwent tryptic digestion after attachment of the aminoethyl-amino-naphthalene (EDANS) chromophore tag. LCMS/MS analysis of a tryptic digest using CID allowed confirmation of all peptides. LCMS/MS analysis using 355 nm UVPD allowed exclusive characterization of the tagged peptide (EDANS-QMEEEAVR) using 3 laser pulses and resulting in an array of diagnostic b/y-type ions.

Conclusions: The differentiation of fragment ions containing the tagged glutamine was readily accomplished using multiple laser pulses, as the abundances of all the fragment ions containing the tag decreased upon exposure to multiple pulses, whereas the abundances of all other fragment ions remain unchanged.

P173

Analytical Comparison of absolute quantification methods to investigate Insulin signaling pathway in OP9 cells

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Topic: Other

Introduction: Targeted proteomics based on mass spectrometry is widely used to help address specific biological questions in an accurate and quantitative way. However, the approaches used for absolute quantification vary in the field, including different methods for the generation of calibration curves and subsequent absolute amount calculation. In this study, different quantification workflows were first evaluated and then compared from three aspects: linear range, recovery factor and LOQ calculation. The most accurate one was then applied to investigate the effect of Tumor necrosis factor-α (TNF –α) on the insulin signaling pathway (ISP). TNF-α is a cytokine linked to regulation of immune response during inflammation. The regulation of proteins on the absolute amount level profiled the effects of TNF-α treatment in normal vs. insulin-sensitive mouse bone marrow stromal cells (OP9).

Methods: The targeted assay was established from our in-house database STAMPS and the Parallel Reaction Monitoring (PRM) mode on a Thermo QExactive HF platform combined with non-linear gradient was used for acquisition.

Results: A calibration curve constructed from amount ratios versus area ratios provided the best accuracy and reproducibility in comparison to the other calibration methods. Based on this method, 113 peptides from 46 proteins in the ISP were successfully quantified. By comparing the results to previously studies, 10 proteins were reported for the first time in the targeted assay by our HR-PRM method. Further our results suggest that some proteins e.g. IRS1 and AKT, are not only regulated on phosphorylation level but also regulated on the protein level.

Conclusions: For the first time we report absolute concentrations of proteins in ISP by PRM. This established method will be applied to investigate how ISP works on normal vs. high fat induced nervous system in near future.

P174

Preservation of Trypsin Activity at Elevated Temperatures for Optimized Proteome Digestion

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Preservation of Trypsin Activity at Elevated Temperatures for
Optimized Proteome Digestion

Topic: Other

Introduction
With the objective of improving digestion efficiency with trypsin for high-throughput proteomics, researchers have employed multiple strategies that point to enhanced enzyme activity. For example, trypsin shows higher initial activity at elevated temperatures, reflected by an increased number of MS identified peptides following a shorter digestion. While maximized initial activity has proven beneficial, it is equally, if not more essential to maintain maximal enzyme activity over the full duration of the digestion period. The present work will determine the variables that influence and optimize initial digestion activity together with enzyme stability, with a goal of providing a high-throughput, high-efficiency approach to proteome digestion.

Methods
Trypsin activity and stability will be measured as a function of temperature, the concentration of organic solvent, surfactants, and calcium. Activity is determined spectroscopically by monitoring the hydrolysis of α-N-benzoyl-ι-arginine ethyl ester (BAEE assay) through time, following preincubation (aging) of the enzyme solution over the range of conditions mentioned.
A dimethyl labeling strategy will be employed to quantify the degree of digestion/ missed cleavage rate of standard proteins by LC-MS/MS.

Results
Preliminary experiments involving BAEE assays demonstrate the controlling influence of temperature and calcium ions on both the activity and stability of trypsin over time. Initial activity is highest at 47°C, showing a three-fold increase compared to 37°C. However, following only 1-hour incubation at 47°C, only 17% of the initial activity remains, dropping below that observed at 37%. The addition of 1-10 mM Ca2+ preserves 70% of the enzyme's activity after 2 h at 47°C, demonstrating the essential role of calcium for tryptic digestion at elevated temperature.

Conclusions
The completed work will provide an improved understanding of the stability of trypsin and thereby impart tools to conduct optimized enzymatic digestion for bottom-up proteome analysis.

P175

Proteomics changes of human ovarian small antral follicles fluid related to upcoming oocyte maturation

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Topic: Other

Introduction: Protein composition of human ovarian follicular fluid (FF) constitutes the microenvironment for oocyte development. Several proteomics studies of FF from pre-ovulatory follicles have revealed insights on oocyte maturation, however, there is a lack of knowledge on changes produced at protein levels in the FF of human small antral follicles (hSAF) related to the upcoming oocyte maturation.

Methods: Using mass spectrometry-based proteomics, we evaluated the protein composition of FF that surrounds oocytes capable to reach metaphase II (MII) after IVM with the protein profile of FF that surrounds immature oocytes. The samples were collected from small antral follicles (size 6.0 ± 1.5 mm) extracted from six women, from which two or three samples were extracted. The comparison was based on both, a multivariate (sPLS-DA) and univariate analyses (t-test).

Results: In total 1,418 proteins were identified from which 35 and 65 proteins were down- and up-regulated, respectively, in FF surrounding oocytes capable to mature. Enriched biological pathways involved processes linked to cell differentiation, follicular development and Wnt signalling. Dysregulated secreted proteins and their correlated non-secreted proteins were mostly enriched in the Metabolism of protein pathway. On the other hand, most of the proteins correlated with one of the secreted frizzled-related proteins were enriched in Transcription pathway.

Conclusions: It was found that changes occur at the protein level in FF from human ovarian small antral follicles related to upcoming oocyte maturation.

P176

Analyzing Assay Redundancy in Metabolomics using Unique Ion Signatures

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Topic: Other

Introduction: The metabolome in complex biological samples provides an understanding of its physiological states. The chemical heterogeneity of metabolites in complex samples requires complementary techniques such as mass spectrometry (MS) to enrich coverage. However, gold-standard targeted, untargeted and data-independent acquisition (DIA) metabolomics workflows are challenged by unambiguous detection based on few fragments. To investigate the problems of assay redundancy and specificity in metabolomics, we used unique ion signatures (UIS) to calculate nonredundant theoretical assays for a given metabolomic background.

Methods: UISn is defined as a set of top n analyte transitions that map exclusively to one metabolite in the metabolome to be analyzed. We simulated different MS methods (MS1-only, MRM, DIA) using the NIST LC-MS library as a background (8274 compounds at collision energy=35). With varying mass accuracy for the precursor m/z window (Q1) and the fragment m/z window (Q3), we compared these methods with regards to unambiguous detection using UIS.

Results: Our simulations show that data generated with DIA using narrow mass windows (25ppm Q1/ 25ppm Q3) outperformed both MS1-only and MRM-based methods with respect to unambiguous detection (54% uniquely identified analytes for MS1-only, 92% for MRM, and 97% for DIA at UIS3). Interestingly, our analysis demonstrates that neither MS1-based extraction at 25ppm accuracy nor a single transition in MRM (0.7Da Q1/0.7Da Q3) is sufficient to uniquely detect the majority of compounds in the NIST library. In addition, the theoretical saturation of a compound's uniqueness is observed based on optimized experimental conditions of matrix complexity and number of transitions (55% uniquely identified analytes for MS1-only, 93% for MRM, and 99% for DIA).

Conclusions: UIS analyses demonstrate the necessity of using both high-resolution precursor and high-resolution fragment ion m/z for unambiguous compound detection. This work also highlights the benefits of DIA for unambiguous compound detection (and quantification) in complex samples.

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Evaluation of immunodepletion strategies applied to the characterization of plasma proteome

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Topic: Plasma Proteomics and Secretome

Introduction: The characterization of low abundance proteins in plasma is a challenge due to the large dynamic range of its proteome. Efforts are currently being made to obtain more information about the plasma proteome since it is ideal for identifying biomarkers. Immunodepletion of the most abundant proteins has been mostly applied in recent studies to access the low abundance proteins. In this work, we evaluated the performance and compared the results obtained from the proteomic analysis of three different immunodepletion strategies.

Methods: The human plasma was submitted to immunodepletion of the 7, 14 (Multiple Affinity Removal Column Human 7 and 14, Agilent) and ~60 (Seppro® SuperMix LC2, Sigma) most abundant proteins. The samples were reduced with DTT, alkylated with iodoacetamide, and digested in two steps with Lys-C and trypsin in S-trap (PROTIFI). The analyses were carried out in an UltiMate 3000 RSLCnano coupled to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific).

Results: We identified 482 proteins in the analysis of undepleted samples, and 652, 773, and 1096 proteins with immunodepletion using the top7, top14, and Supermix columns, respectively. The immunodepletion did not result in many advantages in the identification of proteins related to some types of biological processes such as blood coagulation. Some proteins classes and functions are enriched in a specific approach as angiogenesis proteins in top 7 depletion, inflammatory response in top14, and membrane proteins in the supermix. However, the results from top7 and top14 depletion were very similar regarding the protein functionality, despite the increase in the total number of proteins identified.

Conclusions: The immunodepletion of high abundant proteins from plasma can increase the total number of proteins identified but the decision of each approach to use depends on the aim of the study since each strategy can enrich a specific group of proteins.

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Validated isotope-labeled protein standards for absolute quantification using mass spectrometry

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Topic: Plasma Proteomics and Secretome

Introduction
The preferred approach for absolute protein quantification with mass spectrometry (MS) is addition of stable isotope labeled (SIL) standards, with established concentration, to the samples. The endogenous protein concentration can be determined from the obtained heavy to light peptide ratios, given that the peptides are measured within their linear ranges. Therefore, investigation of the suitability of the tryptic peptides for quantification is necessary.
Quantitative Protein Epitope Signature Tags (QPrESTs) are SIL protein standards with an amino acid sequence (50-150 aa) identical to a part of a human protein and covers several tryptic peptides. The QPrEST standards are added to the sample before tryptic digestion and its peptides are formed in a similar manner as for the endogenous protein.

Methods
QPrEST standards were spiked into human plasma prior to digestion and the samples were analyzed using a targeted MS approach. The QPrEST tryptic peptides were validated according to the CPTAC criteria to certify the analytical performance of the quantification method. Two experiments were performed to determine linear range, limit of detection (LOD), lower limit of quantification (LLOQ) and repeatability. In addition, results for different QPrEST peptides were compared to further verify the data.

Results
Ten investigated QPrEST standards could be successfully validated with at least one peptide fulfilling the CPTAC criteria. Concentrations of the target plasma proteins were calculated based on the heavy to light ratios for the validated QPrEST peptides and the obtained data was verified with at least one additional QPrEST peptide showing similar concentration (±20%).

Conclusions
The results show the successful validation of peptides from ten QPrESTs covering many clinically interesting targets, including several apolipoproteins. This suggests that these QPrEST standards are well suited for use in single-point calibration experiments, enabling a simple workflow for MS-based absolute protein quantification.

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Doublecortin-like kinase 1 modifies small extracellular vesicle cargo and secretion in kinase-dependent manner

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Topic: Plasma Proteomics and Secretome

Introduction: Doublecortin-like kinase 1 (DCLK1) is a putative cancer stem cell marker, a promising diagnostic and prognostic maker for malignant tumors and a proposed driver gene for gastric cancer. Its overexpression in a majority of solid cancers positively correlates with lymph node metastases, advanced disease and overall poorer outcome. DCLK1 has a functional Ser/Thr kinase domain which is important for protein stability and for negatively regulating microtubule polymerization mediated through its tandem N-terminal doublecortin (DC) domains, along which kinesins transport small extracellular vesicles (sEVs) to the plasma membrane.

Methods: sEVs were isolated by ultracentrifugation from conditioned media of parental gastric cancer cell line MKN1 (MKN1par), DCLK1 overexpressing MKN1 (MKN1oe), and small molecule inhibitor DCLK1-IN-1 treated MKN1oe cells (DCLK1oe+inh). A trans-well migration assay induced a 2-fold increase in migration when MKNoe sEVs were added to MKN1par cells compared to MKN1par sEVs. Lastly, a quantitative label-free LC-MS/MS analysis was performed on sEVs secreted by MKN1par, MKN1oe, and MKN1oe+inh, to identify altered protein cargo of sEVs.

Results: The isolated sEVs were positive for exosomal markers Alix and TSG-101. NTA analysis revealed an increase in vesicle size and concentration when DCLK1 is overexpressed. Upon DCLK1 inhibtion, these observations were reversed and similar to parental sEV size and concentration. The quantitative proteomic analysis revealed a total of 381 significantly altered proteins within sEV cargo when DCLK1 is overexpressed. Among those, 61 proteins were significantly altered upon DCLK1 inhibition, suggesting a kinase dependent function for these proteins. Gene enrichment and STRING analysis revealed upregulation of tumor-promoting biological processes.

Conclusion: This data suggest that DCLK1 is responsible for cargo selection and secretion in a kinase dependent manner. The sEVs derived from DCLK1 overexpressing cells are able to increase migration and protein cargo reveals proteins that promote tumor growth and invasion, ECM remodeling and immune evasion.

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Development of plasma depletion method for high throughput plasma analysis by mass spectrometry

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Topic: Plasma Proteomics and Secretome

Mass spectrometry is a powerful technology helping to identify and to quantify hundreds of plasma proteins. However, the large dynamic range of plasma is a major problem for using MS analysis, robust methods to fractionate or enrich lower abundant proteins are required. We developed and optimized a workflow for the analysis of plasma depletion using i) anti-camel antibodies depletion and ii) data acquisition using a high and mid-throughput DIA-MS protocols.

Using TOP14 depletion resin (beads coupled to anti-camel antibodies, High Select™ Top14 Abundant Protein Depletion Resin, Thermo Scientific) in 96 well format, the depletion efficiency (based on protein intensity after DIA acquisition) was determined under varying conditions (e.g. plasma dilution, organic solvents addition, sonication, heat etc). Two LC-MS platforms were evaluated: Phenomenex-Exploris (60min gradient) vs Evosep-Exploris (25min gradient) and optimal loading was determined thanks to loading curve (range 39-2500ng). Reproducibility was determined based on an experiment with 5 replicates per day for 3 days.

A number of plasma pre-treatments improve the depletion efficiency, but the gain was too low compared to the loss of reproducibility (<30% of reproducibility). However, plasma dilution (1:10 in ammonium bicarbonate) increase depletion efficiency (based on intensities for targeted proteins) and a wider protein quantification (+11% compared to non-diluted plasma). Optimal loads were 1250 and 625ng for Phenomenex and Evosep columns, respectively. Phenomenex-Exploris system gave a better proteome coverage compared to Evosep-Exploris platform (+49.8% of quantified proteome) with the same proportion of robustly quantified proteins (≈72% of proteins quantified with CV≤20%).

Our method for plasma depletion can be implemented on an automation system (i7 robot, Biomek) to increase high throughput for both naïve and depleted sample preparation. The robustness and the depletion efficiency of this protocol path the way of MS analysis on plasma sample applied to precision health studies.

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Proteograph™, a multi-nanoparticle-platform, enables deep unbiased plasma proteomics at scale and speed, significantly improving coverage and scalability versus traditional methods

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Topic: Plasma Proteomics and Secretome

INTRODUCTION: Plasma contains protein variants from most tissues and is a rich source of disease biomarkers. However, plasma's large range of protein concentrations necessitates complex workflows that often limit throughput and scalability, are time consuming and expensive, and sacrifice coverage and precision. We have developed an unbiased high-throughput quantitative proteome profiling platform, Proteograph, which leverages the selective protein-nanosurface interactions of nanoparticles engineered with distinct physicochemical properties to provide deep and broad coverage of the plasma proteome at scale. We have compared the Proteograph to common plasma proteomics workflows across a number of parameters including depth, dynamic range, coverage, throughput, and precision and demonstrated reproducible quantification of approximately 2,000 proteins (Blume et al, Nat. Commun., 2020).

METHODS: A direct comparison of the Proteograph to typical workflows, including abundant protein-immunodepletion and high-pH peptide fractionation, was performed using a common EDTA plasma sample. Processed samples were analyzed by micro LC-MS/MS interfaced to a Sciex 6600+ mass spectrometer (MS) operating in data-independent mode. Data were processed using Spectronaut (1% peptide and protein false discovery rates). Coverage, depth, precision, and workflow efficiency were evaluated, with all statistical analyses performed in R.

RESULTS: Proteograph identified more than 1,600 protein groups in this common sample, spanning nearly the full range of abundance in plasma. Compared to standard fractionation workflows, Proteograph captured proteins on average at 10-times lower abundance, outperforming the identification rate of high-pH fractionation fivefold for the two lowest orders of magnitude. Moreover, the Proteograph workflow upstream of MS requires only 7 hours with only 30 minutes of hands-on time and has better precision compared to depletion and fractionation methods, which require multiple days.

CONCLUSION: Proteograph demonstrates superior performance on evaluated measures, making it a robust and efficient platform for unbiased, deep, rapid, large-scale proteomics to quantify thousands of proteins across large numbers of samples.

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Facets of individual-specific, longitudinal plasma proteome profiles

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Topic: Plasma Proteomics and Secretome

Introduction: To gain insight into individual baselines and longitudinal variability of protein levels in circulation, we studied plasma proteomes of 101 individuals by exploratory antibody bead arrays.

Methods: Plasma samples were collected from clinically healthy individuals at four visits during one year[1]. Starting with 1,483 antibodies, we set up a validation scheme based on technical reproducibility and, for available data, correlation to orthogonal methods (Olink, MS and GWAS). In total, 734 proteins were selected for downstream analysis. We performed association tests between genetic variants and protein levels. Weighted gene co-expression network analysis (WGCNA) was applied for identifying modules of co-varying proteins. Linear models were applied across personal plasma proteomes and referenced to the study population mean to identify changes in protein profiles through time.

Results: We found that 61% (447/734) of the targets had a high technical reproducibility of intraclass correlation (ICC) ≥0.8. Focusing on this subset of targets, 80% (359/447) of the proteins were stable across one year with ICC≥0.8. UMAP analysis revealed that each participant had a stable and person-specific plasma proteome profile, which was further retained thought all visits. Comparing genetic data with protein profiles, we identified 15 cis-pQTLs for which protein abundance is regulated at the level of gene expression or protein polymorphism. WGCNA identified that eight modules of 11-242 proteins were part of longitudinally preserved patterns. Two modules were identified with consistent associations to lifestyle-related factors, including low-density lipoprotein, triglycerides and total cholesterol.

Conclusion: Our study highlights individual-specific protein expression, as well as global trends of longitudinally preserved protein profiles and protein groups. Integrated with clinical data, this could allow for defining normal variation in healthy individuals and assist in detecting perturbations or transitions into a disease.

[1]Tebani et al., “Integration of molecular profiles in a longitudinal wellness profiling cohort”, In press Nature Communications.

P183

Mining the prostate cancer proteome using an ion mobility, high throughput approach for large cohort analyses

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Topic: Plasma Proteomics and Secretome

Introduction
Prostate cancer is the second leading cause of cancer deaths for men in the U.S. with around 1 in 9 men being diagnosed with the disease. In order to provide a comprehensive and statistically valid sample set, large cohorts of individuals are required to be sampled. This ultimately provides an analytical challenge, particularly for proteomic focussed research, whereby nanoscale chromatography is routinely adopted. Here, we demonstrate the use of high throughput strategies for proteomic profiling of plasma, derived from prostate cancer individuals. Combined with an ion mobility, data independent acquisition, highly robust, in-depth profiling and quantification of the plasma proteome is highlighted for large cohort analyses.

Methods
Plasma samples (n=509) were depleted prior to reduction, alkylation and trypsin digestion. Samples were separated using microscale chromatography with a sample turnaround time of 25 minutes. The liquid chromatography system was coupled to a Synapt XS mass spectrometer and data obtained using an ion mobility enabled DIA method. Data were processed using a variety of informatic tools and searched with respective databases.
Samples from the cohort were analysed in a randomised manner with sample type and metadata unknown to the instrument analyst. In addition to mass measurement, the instrument was configured to provide peptide Collision Cross Section (CCS) measurements. Samples were assigned to the different groups within the sample set, revealing a significant number of proteins with differential regulation between the various study groups. Proteins occurring in a minimum of two biological replicates and ANOVA $p < 0.05$ were considered as significant. Resulting peptides were further analysed using multivariate analysis, indicating separation of the groups. Peptide CCS values of peptides found to be common in a significant number of the samples showed great consistency (<2% CV) across the analysis. Curated data was then subjected to pathway analysis confirming their biological significance.

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Discovery of Blood Biomarkers for Alzheimer's Disease based on Surface Accessibility in Covalent Labeling Mass Spectrometry

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Topic: Plasma Proteomics and Secretome

Introduction: To unlock the full potential of disease-modifying treatments for Alzheimer's disease, which are expected to be most efficacious at the disease's earliest and mildest stages, supportive biomarker information is necessary. For practical reasons, blood-based biomarkers that could signal the mild cognitive impairment (MCI) stage or even earlier would be ideal.

Methods: A structural proteomics approach that incorporated covalent protein painting (CPP) method and mass spectrometry quantified the relative accessibility of small molecules as molecular paints to tightly coat the surface of protein-protein native complexes. Plasma samples were analyzed from 50 probable Alzheimer's disease (AD) subjects, 50 subjects with MCI, and 50 normal control (NC) subjects who did not progress to MCI or dementia. To construct an accessibility classifier model using machine learning algorithms, five-fold cross-validation was repeated 200 times in training dataset (35 samples in each group) and independently assessed the performance of the classifier model in test dataset (15 samples in each group).

Results: A three-feature model was used for random forest comparison: NC versus MCI had an area under the receiver operator characteristic curve (AUROC) value of 0.942, sensitivity of 0.867 and specificity of 0.733 in the test dataset. A four-feature model was used for deep learning comparison of MCI versus AD participants; this was established and demonstrated high accuracy (testing AUROC = 0.947, sensitivity = 0.733, and specificity = 0.933). In an additional verification study, the classifier model had similar accessibility in the age groups (<75 y and ≥75 y), unlike NCI vs. MCI and MCI vs. AD. Consequently, the classifier model is not a general aging biomarker, but a predictor of MCI and AD.

Conclusions: The study of conformational changes in proteome through this CPP method unbiasedly highlights putative and novel biomarker candidates for AD pathology to further validate with multi-site and large-cohort scale.

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Development of a modular workflow for precise DIA-MS analysis of three blood proteomes

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Topic: Plasma Proteomics and Secretome

Introduction
MS based proteomic studies have demonstrated great potential through the discovery of many candidate circulating disease markers. However, it is widely accepted that robust discovery pipelines are required to translate potential biomarkers into clinical utility. We developed a workflow that allows flexibility between high and mid-throughput analysis for three blood based proteomes.

Methods
Optimized for each step from sample processing and DIA-MS data acquisition on an Exploris 480 (Thermo), we established a high-throughput, 25 min method using an Evosep One and mid-throughput 60 min method which uses a Phenomenex Luna Omega C18 column and an Ultimate 3000. Digestions were performed on an automated workstation (Beckman i7). Using TOP14 depletion resin (Thermo) 10 ul of plasma was depleted (n=5) then lyophilized prior to digestion. Native plasma (5ul, n=5) and whole blood (10 ul, absorbed onto mitra device, n=5) were denatured with 30 % TFE and 40 mM DTT, alkylated with 10 mM IAA and digested with trypsin. Samples were prepared to assess reproducibility on 3 separate days. Data processed using Xaclibur, MAP DIA and plasma pilot.

Results
Our optimized workflow supported the quantification of 200 (25 method) and 504 (60 method) plasma proteins. Median Inter-day Cvs ranged from 8-10 % (25 method) and 10-13 % (60 min method) while median intra-day Cvs were 18 % (25 method) and 24 % (60 min method). For whole blood a 366 (25 method) and 578 (60 method) proteins were quantified. Median Inter-day Cvs ranged from 15-17 (25 method) and 10-13 (60 min method) while intra-day Cvs were 20% (25 method) and 18 % (60 min method). Depletion data is in progress.

Conclusion
Our workflow represents an excellent pipeline to support robust multiprotein biomarker studies. We envisage that implementation on a large scale will facilitate the translation of candidate markers into clinical use.

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High throughput quantitative plasma proteomics on the timsTOF pro platform

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Topic: Plasma Proteomics and Secretome

Introduction
Plasma is an easily accessible source for monitoring proteins and deriving the physiological state of an individual. However, the dynamic range of the plasma proteins has remained daunting and a driving force for technological advances. For large scale clinical proteomics plasma is typically analyzed without any depletion and in an unfractionated manner. Here we provide an update on the performance of timsTOF Pro platform for different gradient lengths.

Methods
Pooled plasma was purchased from Sigma and digested using the iST sample preparation kit (Preomics, Germany) (. Purified peptides were then separated on a 25 cm Aurora column (IonOptiks, Australia) using a NanoElute syste (Bruker Daltonik, Germany) coupled online to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro, Bruker Daltonik). Data were acquired with both DDA and DIA methods and raw data were processed using MaxQuant or Spectronaut software.

Results
Plasma proteome were measured in 25, 45 and 60 minute gradients both in DDA and DIA. Match between the runs feature available in MaxQuant was used in order to minimize the missing value problem. The DIA data were processed in Spectronaut with a library constructed using depleted plasma peptides. The library consisted of more than 1000 proteins. Using the library more than 400 proteins could be quantified in 25 minute gradients. DDA runs resulted in more than 500 protein quantified in 45 minute gradients.

Conclusion
Both DDA and DIA based approaches offer reasonable depth of proteome quantitation using timsTOF pro platform and indicates that clinical studies using plasma are becoming feasible.

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Plasma Protein Biomarker Identification for Monitoring Molecular Changes in Smokers, Former Smokers, and E-Vapor Product Users Relative to Never Smokers

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Topic: Plasma Proteomics and Secretome

Introduction

Inhaled toxicants present in cigarette smoke accelerate and exacerbate inflammation and oxidative stress. Over time, these processes increase the risk of developing respiratory and cardiovascular diseases. Developing alternative tobacco or nicotine products that have the potential to reduce the risk for smokers who would otherwise continue to smoke is one approach for addressing these health concerns. Electronic-vapor products (EVP) are proposed as potential reduced-risk products; however, their impact requires in-depth investigation. This study aimed to examine molecular profiles and biological networks that are perturbed by smoking, reversible upon smoking cessation, and largely unknown with respect to EVP use, to identify new biomarkers.

Methods

Here, we present the plasma protein profiling data from this study. About 200 plasma samples were obtained from smokers (CS), former smokers (FS), and EVP users and compared with those from never smokers (NS). The samples were processed in randomized order by using Sample Preparation Kit Pro (Biognosys, Switzerland) and further analyzed on a UPLC coupled to a Q-Exactive HF by using the data-independent acquisition (DIA) mode after PQ500 spike-in .

Results

To our knowledge, this study is the first human research study to use PQ500 spike-in combined with DIA analysis of human plasma. This approach allowed us to accurately quantify 140 proteins across the entire dataset by using Spectronaut software.

Specific molecular changes associated with smoking and cessation were detected. The abundances of 14, 5, and 0 proteins were significantly different in plasma in the CS vs. NS, FS vs. NS and EVP vs. NS group comparisons, respectively.

Conclusions

Integration of these data with other omics measurements as well as with demographics and EVP usage history is expected to support potential risk reduction in adult smokers who switched to EVP or quit smoking.

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A novel workflow for comprehensive profiling of plasma exosomes using data-independent acquisitions

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Topic: Plasma Proteomics and Secretome

Introduction: Exosomes are membrane-bound extracellular vesicles (EVs) that facilitate the transport of cargo to proximal and distant cells in the body. Exosomes remarkably impact cellular signaling, and hence the protein cargo may reveal promising biomarker candidates for many diseases and aging processes. One of the major challenges in the clinical applications of exosome 'omics' analysis lies in the enrichment efficiency and purity of exosomes from plasma. Here, we report a plasma exosome study including analysis of proteins and miRNA from young and aged individuals.

Methods: Human plasma from young (20-26 years) and old (60-66 years) individuals were obtained from the 'Blood Centers of the Pacific'. Plasma exosomes were isolated by sequential size-exclusion chromatography (SEC) and ultrafiltration (UF). Quality control analysis of the isolated exosomes confirmed high exosome purity. Exosome protein lysates were digested and subjected to high-resolution mass-spectrometry. Data-dependent acquisitions (DDA) were performed to generate a deep-library for plasma exosomes which was subsequently used as reference for the data-independent acquisitions (DIA) for comprehensive protein identification and quantification of exosomes from young and old individuals. The exosome miRNA profiles of the same samples were generated by next-generation sequencing.
Results: We generated a deep proteome library consisting of ~2,300 exosome proteins. In this pilot study, we identified and quantified 1,208 exosome proteins of which 204 proteins were significantly altered comparing old with young individuals ($Q<0.05$ and >1.5-fold change). Exosome miRNA analysis of the same samples showed that 6 out of 331 miRNA were significantly altered in old vs young ($p<0.05$, >1.5-fold change), and 88 miRNA were unique to old individuals. Several protein pathways were upregulated in the old, including acute inflammatory response and antioxidant. Defense response, intermediate filament, and cytoskeleton pathways were downregulated.

Conclusion: DIA and RNAseq approach to explore age-specific changes in exosomes for biomarker discovery for aging and aging-related diseases.

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In-depth serum proteomics identifies potential biomarkers for papillary thyroid carcinoma diagnostics

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Topic: Plasma Proteomics and Secretome

Introduction
Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy. Preoperative diagnosis is inconclusive in up to 30% of thyroid nodules which may result in substantial over treatment and a lifelong thyroxine-replacement therapy. Therefore, identification of biomarkers of malignancy minimal invasively is necessary to improve the early PTC detection and subsequent management.

Methods
In this pilot study, we screened 54 serum samples from 27 PTM patients before and after thyroidectomy and 27 serum samples from healthy controls using an in-depth serum proteomics platform with customizable antibody microarrays and data-independent acquisition mass spectrometry (DIA-MS) which enables the quantification of over 1,000 serum proteins across ~10 orders or magnitude (Hou et al., 2020; Xu et al., 2019).

Results
The results identified 210 differentially-expressed proteins in PTC patients compared to healthy volunteers by statistical analyses ($p \leqq 0.05$), which are involved in a variety of biological processes, such as blood coagulation, inflammation, apoptosis and humoral immune response signaling pathways. Before and after surgical removal of the tumor, there is a spectrum of differential proteomic signatures which is dissimilar to alterations of PTC patients versus healthy volunteers.

Conclusions
Our data provide valuable proteomic information for the changes of inflammation and coagulation signaling in PTC, and potential biomarkers for the clinical validation in future.

Keywords
proteomics, papillary thyroid carcinoma diagnostics, mass spectrometry, data-independent acquisition, circulating biomarkers

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P190

A Pilot proteomics study reveals differentially protein expression related to testosterone and gonadotropin changes in a short-term controlled Healthy Humans

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Topic: Plasma Proteomics and Secretome

Introduction: Testosterone deficiency afflicts approximately 30% of men aged from 40-89 years. The causality of the relationship between low testosterone and metabolic diseases is unclear. To determine the influence of alterations in testosterone concentration on the molecular profile in healthy individuals, a controlled and short-term human cohort was recruited.

Methods: We analysed plasma samples from thirty healthy male volunteers (19-32 years of age) with pharmacologically-induced testosterone deficiency. Blood samples were collected under three conditions: baseline, low testosterone, and restored testosterone. As a proof-of-concept, a sample pooling approach was used to obtain one pool per condition, with each pool consisting of 30 individual samples. We evaluated the changes at proteome level by pooling the 30 samples by time point (A, B, and C). The three pools were processed and analysed by liquid chromatography mass spectrometry using data-dependent acquisition. The data was subject to enrichment analysis based on the 2D annotation enrichment algorithm created by Cox and Mann(Cox and Mann 2012). The Human Proteome Map was used to determine the tissue distribution of the differentially expressed proteins.

Results: The results obtained indicated that it was indeed possible to reveal significant differences at the protein level across the three groups. Although this short-term human model is a controlled study with healthy subjects, the approach is considered a proof-of-concept for a much larger, highly-promising clinical evaluation. This is of particular importance because the causality of the relationship between low testosterone and metabolic diseases is not well-known.

Conclusions: Under the influence of altered levels of gonadotropin and testosterone in blood, the three conditions studied here were readily separated according to protein content and expression profile. The approach was successful but could benefit further from a deeper level of scrutiny. This would include the analysis of individual samples to take such differences into account.

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The accessible plasma proteome with Top 7 immunodepletion spin columns. A comprehensive study.

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Topic: Plasma Proteomics and Secretome

The presence of high abundant proteins in plasma masks the detection of low abundant proteins during mass spectrometry analysis. The immunodepletion of the most abundant proteins has often been used to overcome this analytical problem and protein identifications ranging from hundreds to thousands have been reported. Several depletion systems are commercially available; however, recent studies lack analysis of the confidence, reproducibility, and accessible proteome after depletion. Here, we present the accessible plasma proteome after Top 7 immunodepletion with spin columns in several plasma samples. Consistently, around 100% of proteins are added after depletion and this number increases up to 170 % when peptide fractionation is performed. The inter and intra-coefficient variation between columns was on average lower than 10%, as was explored by MRM analysis of 17 proteins throughout the dynamic range of protein identification.

P192

Understanding cell development in 3D culture models and secretome characterization by proteomics

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Topic: Plasma Proteomics and Secretome

Introduction

Cell-derived secretome has gained attraction in recent years as a very useful tool in regenerative therapies. It circumvents many of the problems associated with the immune response of the host tissue against the implanted cells. The usual standard 2D culture conditions do not allow for tailoring the secretome so that it matches the needs of the regenerative therapy. 3D matrices with synthetic backbones have emerged as attractive candidates as they provide precise control on stem cell fates via customizable properties.

Methods

Adipose-derived stem cells (hASCs) were seeded in matrices with different mechanics and biofunctionalization at the same polymer concentration. Luminex and ELISA were used to identify markers secreted by hASCs to their conditioned media (CM). We next used quantitative proteomics analysis based on Tandem Mass Tag (TMT) isotopic labelling of the secretome from the hASCs to fully characterize the set of secreted markers.

Results

Luminex and ELISA revealed that cells in 3D adhesion peptide free matrices secreted increased levels of IL-10 and their conditioned media (CM) induced significantly faster wound closure than 3D matrices with adhesion peptides and 2D culture on tissue culture polystyrene (TCPS). IL-10 depletion using anti-IL-10 antibodies reduced the effect of the CM. Nevertheless, IL-10 addition to cell culture media, did not induce the same change in wound closure speed as the CM. TMT was used to, on the one hand, fully characterize the set of secreted markers and elucidate which of them is inducing the synergistic effect observed with IL-10. And on the other hand analyze the whole protein extracts of the hASCs.

Conclusions

Proteomics analyses revealed an increase in protein digestion and absorption and ECM-receptor interaction KEGG pathways when cells were cultured in 3D. In contrast, 2D cultured cells showed higher expression levels of proteins involved in ECM organization and cell senescence.

P194

Characterisation of novel synaptic substrates of the deacetylase Sirtuin-2

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Topic: Post-Translational Modifications

Introduction: Recent studies have identified a new role for non-histone lysine acetylation in the regulation of synaptic protein stability. Bioinformatic analysis of existing proteomic data shows that the majority of synaptic protein are acetylated and acetylation is particularly enriched in the synapse with a significant association with neurophysiological functions (1-2). The function of a limited number of synaptic proteins has been investigated. The AMPA receptor and Arc (Arg3.1) are deacetylated by Sirtuin-2 (SIRT2) on sites that can also be ubiquitinated; this balance between acetylation and ubiquitination regulates their function in synaptic plasticity, learning and memory (1, 2). SIRT2 is expressed highly in the brain and is associated with many neurodegenerative diseases. However, the substrates and regulatory role of SIRT2 is poorly understood at the synapse.

Methods: Here we have sought to identify putative SIRT2 substrates using quantitative analysis of the acetylome using mass spectrometry analysis of immuno-enriched acetylated peptides from SIRT2 knockout and wild type brain tissue.

Results: Using this strategy, we have identified 2,267 unique acetylation sites on 1,553 acetylated proteins. Among them, 246 sites have significantly higher acetylation levels in SIRT2 knockout brain tissues and represent putative SIRT2 substrates. 40% of these sites are also ubiquitinated indicating a substantial degree of crosstalk between these PTMs and highlights sites that may be important regulatory switches at the synapse.

Conclusions: Our data identify novel SIRT2 substrates that will advance the understanding of the role of SIRT2 in neurodegenerative disease and help to link those substrates with specific downstream processes.

P195

Mapping PTM data from UniProtKB to ChEBI facilitates integration of proteomics and metabolomics

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Topic: Post-Translational Modifications

Introduction

Proteomics provides a powerful means to detect and study post-translational modifications (PTMs), which play a key role in the regulation of protein function. Here we describe a project to map all PTMs described in UniProtKB (www.uniprot.org/docs/ptmlist) to the ChEBI ontology of small molecules and derivatives (www.ebi.ac.uk/chebi/). UniProtKB uses ChEBI to describe small molecules such as enzyme cofactors as well as enzymatic reactions, the latter through Rhea (www.rhea-db.org), a resource of biochemical transformations built on ChEBI. Mapping PTM descriptions in UniProtKB to ChEBI will further enhance the links between small molecules and proteins in UniProtKB and will facilitate the integration of PTM data with knowledge of enzymes and biochemical networks.

Methods

ChEBI describes small molecules and derivatives thereof, including modified amino acid residues in proteins, which are termed “groups”. To create the UniProtKB PTM-ChEBI mapping, we review each PTM described in UniProtKB and map it to the appropriate ChEBI group; when no such ChEBI group is available, we first curate the required group into the ChEBI ontology.

Results

We have begun by mapping the most common PTMs in UniProtKB to ChEBI groups, mapping 252 UniProtKB PTMs to ChEBI to date, representing 265000 sites annotated in UniProtKB/Swiss-Prot. The UniProtKB PTM – ChEBI mapping is updated at each UniProt release and is provided at www.uniprot.org/docs/ptmlist.

Conclusions

We now provide explicit machine-readable representations of PTMs and their chemical structures in UniProtKB using functional groups from the ChEBI ontology. This work provides enhanced support for the integration of proteomics and metabolomics using UniProtKB – linking knowledge of PTMs with metabolic networks and enzyme function through ChEBI. Future work will focus on completing the mapping of all PTMs and on extending the UniProt entry to include links from PTMs to ChEBI.

P196

Precision Mapping of O−Linked N−Acetylglucosamine Sites in Proteins Using Ultraviolet Photodissociation Mass Spectrometry

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Topic: Post-Translational Modifications

Introduction: O-linked N-acetylglucosamine (O-GlcNAc)is unique from other common forms of protein glycosylation in several respects: (1) it occurs co-translationally within the nucleus of the cell, (2) it occurs post-translationally in the cytoplasmic compartments of the cell, and (3) it often occurs at sub-stoichiometric amounts on a protein with a distribution similar to that of Ser(Thr)-O-phosphorylation. Despite its central importance as a regulator of cellular physiology, identification and precise mapping of O-GlcNAc post-translational modification (PTM) sites in proteins by mass spectrometry (MS) remain a considerable technical challenge. Here we report the use of an HCD-triggered 193 nm ultraviolet photodissociation (UVPD) data-dependent method for the precise localization of O-GlcNAc sites.

Methods: Glycoproteins were digested in-gel with trypsin and separated by nanoLC interfaced to an Orbitrap Lumos MS equipped with a 193 nm excimer laser for UVPD. A tandem mass spectrum (MS/MS) UVPD event was triggered using a targeted mass list of HexNAc oxonium ions generated using collisional activation dissociation (HCD). UVPD spectra were analyzed using ProteinMetrics Byonic and validated using ProSight Lite. The main scoring metrics used to gauge the confidence of identified glycopeptides are the PCS score, Delta Mod Score, and the PEP-2D score, respectively.

Results: The advantage of this method was twofold: the HCD MS/MS spectrum yielded characteristic b- and y-type sequence fragment ions that generated reliable peptide sequence information (but with loss of the glycan moiety), and UVPD MS/MS spectra yielded extensive fragmentation with retention of the glycan group. This method provided unambiguous site-localization of glycan moieties on previously unmapped O-GlcNAc sites on TAK1-binding protein-1 and polyhomeotic-proximal chromatin protein. The pre-scanning feature of this two-stage method offered a fast precursor screening rate without the burden of continuous MS3 data acquisition.

Conclusions: A triggered HCD/UVPD was developed to precisely map protein O-GlcNAcylation sites, complementary to existing HCD- and ETD-based methods.

P197

Multi-pathway signaling analysis using a synthetic phosphopeptide panel, standardized sample preparation kits and SureQuant internal standard targeted quantitation

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Topic: Post-Translational Modifications

Introduction: Quantitative measurements of signal transduction pathway proteins and their post-translational modifications such as phosphorylation, are necessary for classifying disease states and uncovering novel signaling mechanisms. We have developed a SureQuant internal standard (IS)-triggered targeted strategy using a pool of phosphopeptide reference internal standards and SMOAC (Sequential enrichment of Metal Oxide Affinity Chromatography) to purify and quantify phosphorylation abundance. Specific phosphopeptide standards were chosen representing phosphosites from several different pathways including EGFR/HER, RAS-MAPK, PI3K/AKT/mTOR, AMPK, death and apoptosis, and stress (p38/SAPK/JNK) signaling. The proposed turnkey workflow enables reliable targeted quantitation for routine phosphoproteomics of biologically relevant phosphorylation sites.

Methods: The workflow leverages a pool of 146 AQUA heavy-isotope phosphopeptides from 89 signaling proteins. Cancer cell line samples were processed using EasyPep™ Maxi MS Sample Prep Kit. Cell digests spiked with phosphopeptide standards were subjected to SMOAC procedure, i.e., sequential Hi-Select™ TiO2 and Fe-NTA phophopeptide enrichment. Eluents were combined before LC-MS analysis on Orbitrap Exploris 480 mass spectrometer. A novel SureQuant targeting method was utilized, enabling real-time confirmation and high-sensitivity measurement of endogenous targets. Data analysis was performed with Skyline software.

Results: In this study, we developed a SureQuant targeted assay based upon 146 AQUA heavy-isotope phosphopeptide standards (covering 158 phosphosites, distributed on 96 serine, 26 threonine and 36 tyrosine residues). With an optimized IS-triggered SureQuant method phosphopeptides were reliably quantified in the SMOAC enriched cell line digests. Various MS acquisition approaches including DDA and PRM were compared with SureQuant to evaluate relative performance differences. Endogenous phosphopeptides could be quantified at 10X lower levels than PRM, to amol/µg levels. This targeted phosphopeptide assay coupled with SMOAC method and novel MS acquisition approach provided excellent quantitation, specificity and selectivity for signaling pathway analysis.

Conclusions: SureQuant targeted analysis allows reproducible, routine and simultaneous quantitation of functionally relevant phosphorylation sites spanning multiple signaling pathways.

P198

Mass spectrometric quantification of histone lactylation marks in human melanoma cells

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Topic: Post-Translational Modifications

Introduction

The Warburg effect, originally describing augmented glycolysis and elevated production of lactate in cancer, is associated with diverse cellular processes (e.g., angiogenesis, hypoxia, macrophage polarization, and T-cell activation) and many diseases (e.g., neoplasia, sepsis, and autoimmune diseases). Lactate, a compound generated during Warburg effect, is known as an energy source and metabolic byproduct. However, its non-metabolic functi-ons in pathophysiology remain unknown, representing one of the major questions in biomedicine. We recently showed that lactate is a precursor for a new protein modification and epigenetic pathway, lysine lactyltaion (Nature, 574:575-580, 2019). We will report here our quantitative proteomics works on determining changes of Kla levels in human melanoma cells in response to a genetic mutation and a clinical drug, vemurafenib.

Preliminary Data

We firstly treated both A431 and A375 cells with different doses of vemurafenib to examine the impact of the drug on histone Kla levels. Histone Kla but not lysine acetylation (Kac) was markedly decreased in BRAF V600E mutated cells (A375) but not in BRAF wildtype cells (A431) after 24 hours' treatment of vemurafenib (PLX4032). We then quantified the decrease of histone lactylation marks in response to vemurafenib by SILAC labeling and mass spectrometry. We identified and quantified 23 histone marks bearing lysine lactylation in melanoma A375 cells. We analyzed the changes of histone Kla as well as acetylation marks. Consistent with Western blot data, vemurafenib has little impact on lysine acetylation levels on core histones. In contrast, vemurafenib dramatically reduced levels of histone Kla marks at N-terminal tails but not at the global regions of core histones. Our data suggest that histone Kla likely contributes functions of anti-tumor effects of vemurafenib. We will carry out the same experiment in A375 cells.

Novel Aspect

Quantification of histone Kla marks in melanoma cells in response to a BRAF inhibitor.

P199

Epigenetic analysis of histone modifications by mass spectrometry

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Topic: Post-Translational Modifications

The DNA in eukaryotic cells is packed in chromatin with nucleosomes as basic unit. Nucleosomes are composed by octamer of four histones, and the global chromatin structure is altered by histone post-translational covalent modifications. Several types of histone modifications are well known such as acetylation, methylation, phosphorylation, and ubiquitination that play a role in the regulation of transcription activity. Histone modifications dysregulation as well as disruption of chromatin remodeling machinery play a fundamental role in many pathologies and cellular mechanisms. The analysis of histone modifications with standard mass mapping procedures is complicated by the highest occurrence of basic residues maily in the regions interested by the modifications (methylation and acetylation). We developed a methodology based on limited proteolysis coupled to MALDI-MS to achieve a complete sequence coverage; then we focused on H4 lysine acetylation and by LC-MSMS and ion extract procedures, we got a relative quantification of the modification.

Once optimized the procedure on standard chicken core histones, the methodology was applied to the investigation of H4 acetylation state in mouse embryonal stem cells treated with DMSO and Trichostatin A(TSA), respectively. TSA inhibits histone deacetylases indicing cell differentiation. By employing the present procedure, we were able to identify the acetylated lysines and quantify the variation of the levels of acetylation occurring in the two different conditions.

P200

Carcinoembryonic antigen glycosylation: revealing novel features of human glycosylation and cancer origin specificity

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Topic: Post-Translational Modifications

Introduction: Carcinoembryonic Antigen (CEA) is a biomarker strongly associated with tumour progression and metastasis. Even though N-glycans make up ≥50 % of the entire CEA molecule, current knowledge on CEA specific glycosylation in health and disease is scarce.

Methods: We used an in-depth glycomics and glycoproteomics workflow including porous graphitzed carbon nano-LC ESI MS/MS next to various LC-ESI MS/MS based glycoproteomics techniques to investigate the glycosylation of CEA purified from four different body sources: human colon cancer (cell line and tissue), tissue from liver metastasis of colon cancer and ascites fluid. TCGA data was used to correlate glycosyltransferase gene with CEA expression.

Results: We show for the first time in an in-depth glycomics and glycoproteomics study that the over 270 different N-glycans identified exhibited antenna fucosylation and sialylation features that allowed a clear CEA body origin assignment. Colon-derived CEA carried a hitherto not described, hexosylated bisected GlcNAc glycoepitope. All analysed CEAs contained a 29th site of N-glycosylation on Asn71, located within a non-canonical 71N-R-Q73 sequence motif critical for CD8 binding. Correlation analyses of CEA and glycosyltransferase genes across the TGCA dataset revealed that CEACAM5 and B4GALNT3 expression levels were indicative for survival prediction.

Conclusions: Our results open novel opportunities to understand CEA function, its role as a cancer marker but also reveal hitherto unknown aspects of glycobiology.

P201

R2-P2 rapid-robotic phosphoproteomics enables multidimensional cell signaling studies

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Topic: Post-Translational Modifications

Recent developments in proteomics have enabled signaling studies where >10,000 phosphosites can be routinely identified and quantified. Yet, current analyses are limited in throughput, reproducibility, and robustness, hampering experiments that involve multiple perturbations, such as those needed to map kinase-substrate relationships, capture pathway crosstalks, and network inference analysis. To address these challenges, we introduce rapid-robotic-phosphoproteomics (R2-P2), an end-to-end automated method that uses magnetic particles to process protein extracts to deliver mass spectrometry-ready peptides for total proteome and phosphoproteome analyses. R2-P2 is robust, versatile, high-throughput, and achieves higher sensitivity than classical protocols. To showcase the method, we applied it, in combination with data-independent acquisition mass spectrometry, to study signaling dynamics in the mitogen-activated protein kinase (MAPK) pathway in yeast. Our results reveal broad and specific signaling events along the mating, the high-osmolarity glycerol, and the invasive growth branches of the MAPK pathway, with robust phosphorylation of downstream regulatory proteins and transcription factors. Our method facilitates large-scale signaling studies involving hundreds of perturbations opening the door to systems-level studies aiming to capture signaling complexity.

P202

“The impact of arginine-methylation in phase separation and RNA-binding proteins dynamics in cancer”

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Topic: Post-Translational Modifications

Introduction. Arginine (R) - methylation is a post-translational modification (PTM) that occurs not only on histones but also on several non-histone proteins modulating protein-protein, protein-RNA as well as protein-DNA interactions. Protein R-methyltransferases (PRMTs), enzymes responsible for R-methylation, are frequently over-expressed in several types of cancer. Previous results collected in our group highlighted that RNA-binding proteins (RBPs) are the largest category of R-methylated non-histone proteins. R-methylation of RBPs seems to be crucial for the dynamic regulation of membrane-less organelles (MLOs), transient aggregates that are composed in most of the cases by RBPs and RNA and that emerged as a result of liquid-liquid phase separation (LLPS).

Methods. As cancer model we used U2OS osteosarcoma cell line as it represents an optimal cellular system to investigate subcellular localization by both biochemistry/proteomics strategies and immunofluorescence. Thanks to a novel proteomic approach combining standard quantitative MS-proteomics methods with orthogonal organic phase separation (OOPS) strategy to isolate RBPs-RNA complexes, we profiled changes in RBPs-RNA binding upon R-methylation modulation. Validation were performed both by western blot and immunofluorescence (IF) analysis of candidate RBPs following drug perturbation.

Results. Preliminary results suggest that R-hypomethylation induces by MS023 treatment (the inhibitor of PRMTs type I) modifies the capability of some RBPs to bind the RNAs. IF validation suggested that R-hypomethylation could be responsible for LLPS of candidate RBPs emerged from proteomic analysis.

Conclusion. Taken together, our results suggest that the regulation of R-methylation is a mechanism through which RBPs change their RNA-binding capability and their subcellular localization, which -in turn- could induce MLO formation.

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P203

PASEF-DDA enables deep coverage single-shot phosphoproteomics and ion mobility-based elucidation of phosphosite isomers

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Topic: Post-Translational Modifications

Introduction: Coeluting and isobaric phosphopeptide isomers are often impossible to resolve in classical MS/MS analyses. Ion mobility spectrometry (IMS) enables their separation based on their collision cross section (CCS), as the position of the phosphogroup affects the ion geometry in the gas phase. Parallel accumulation serial fragmentation data-dependent acquisition (PASEF-DDA) on the timsTOF Pro mass spectrometer allows the application of IMS on large scale phosphoproteomic studies.

Methods: Tryptic phosphopeptides from two osteosarcoma samples were enriched in three replicates from 1 mg lysate each by TiO2. Enriched phosphopeptide samples were separated within 100 min (2 to 35% B, B: 0.1% FA in ACN, 400nL/min flow rate) on a reversed-phase C18 column with an integrated CaptiveSpray Emitter (25cm x 75µm, 1.6µm, IonOpticks). After ESI ionization, peptides were analyzed using timsTOF Pro with PASEF enabled at 120Hz. Trapped ion mobility accumulation and elution times were synchronized at 166ms. In addition to high resolution (40,000) accurate mass (<10ppm) the mass spectrometer records mobility (1/K0), and with charge state and m/z deciphers CCS.

Results: From single LC-MS analyses of enrichment triplicates, 238,727 total peptide spectrum matches (PSMs) and 191,244 PSMs for phosphorylated peptides were obtained from database search. We identified 39,058 peptides (1%FDR) in total containing 29,803 phosphorylated species. Around 70% of all peptides were identified in each of the enrichment triplicates. The identified phosphopeptides comprised 5,037 isobaric positional isomer pairs. 1,097 of these isomer pairs were separated using IMS. Among these 50% could not be resolved by LC. Phosphoproteome information was used for the identification of hyperactive kinases using Integrative Inferred Kinase Activity (InKA) analysis.

Conclusion: Our data shows that a simple TiO2 enrichment method in combination with PASEF acquisition on the timsTOF Pro allows to obtain a previously unmatched quantity and quality of phosphopeptide information without offline prefractionation.

P204

Evaluating S-Trap, SP3, and In-Solution Digestion Methods for 10-Fold Reduced Sample Requirements in Ubiquitin Proteomics

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Topic: Post-Translational Modifications

Introduction: Protein ubiquitination regulates signaling by triggering protein degradation. Digesting modified proteins with trypsin results in a branched lysine-diglycine (KGG) motif than can be enriched with a motif-specific antibody. Historically, ubiquitin proteomics studies required 5-15mg of protein input prepared by lysis in urea and in-solution digestion (ISD) followed by a time-consuming C18 solid phase extraction. While this method yields thousands of KGG sites, the high input requirements limit the biological questions that can be addressed. Additionally, urea can cause unwanted carbamylation.

Methods: Three alternative digestion methods, each with its own lysis buffer, were investigated for total proteome coverage, effects on KGG enrichment, and overall ease of use. Two methods, SP3 and S-Traps, used detergent-based lysis buffers and avoided the C18 cleanup step for significant time and labor savings. The third method replaced urea with sodium deoxycholate (SDC) but retained the ISD and C18 cleanup.

Results: In triplicate analyses of Hek293 cells, the S-Trap lysis buffer, 5% SDS, extracted 10-20% more protein per cell than any other buffer. After digesting equal cell numbers equivalent to 1mg of protein, S-traps matched urea-ISD for peptide and protein peak area, with few proteins showing significant ($p<0.05$) fold changes. SP3 and SDC-ISD showed poorer recovery, with the median protein 60-70% as abundant as in the urea controls. KGG enrichments from both the urea-ISD and S-trap preparations yielded 10,000 modified peptides, 67% more than the SP3 and SDC-ISD preparations. SP3 and SDC-ISD methods were compatible with KGG enrichment, but the input must be adjusted to account for poorer protein extraction per cell lysed.

Conclusion: Lysis in 5% SDS and digestion with S-Traps improved protein yield over urea-ISD, SP3, or SDC-ISD workflows. This enabled KGG enrichment from 80-93% less input material and saved a full day of hands-on time in comparison to the old workflow.

P205

Glycopeptide profiling in a trapped ion mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro)

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Topic: Post-Translational Modifications

Introduction

Glycosylated peptides present unique analytical challenges for LC-MS/MS with respect to chromatographic separation, electrospray ionization, and structural elucidation by collision induced dissociation experiments. Here we present optimization results for glycopeptide analysis on the timsTOF Pro instrument operated in PASEF mode in combination with C18RP liquid chromatography.

Methods

Glycopeptides from glycoprotein standards and blood plasma were separated by reversed phase liquid chromatography with online analysis in a trapped ion mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro, Bruker Daltonics). RP LC conditions were evaluated to optimize glycopeptide separations. Electrospray ionization conditions ,TIMS, and ion optics were tuned for optimal transmission of glycopeptide precursors and fragment ions. Low- and high collision energy conditions were optimized for glycan- and peptide fragmentation experiments, respectively. Impact of different PASEF data acquisition strategies and parameters on glycan- and peptide-moiety identification rates were evaluated using GlycoQuest, MASCOT, and prototype glycoproteomics software from BSI.

Results

We selected a mobile phase composition of 0.1% formic acid with 0.01% TFA which balances chromatographic performance with ion suppression effects. The addition of 0.01% TFA also preserved co-elution of glycoforms by minimizing interactions of sialic acids with the stationary phase. Co-elution of isobaric and near-isobaric glycoforms of the same peptide due to TFA addition is an advantage for accurate determination of glycoform stoichiometries but might lead to chimeric MS/MS spectra acquisition. We currently evaluate to what extend the orthogonal IMS and MS precursor selection prevents chimeric spectra acquisition. Collision energies (CE) in the timsTOF Pro were scaled relative to precursor mobility values (1/K0) rather than m/z and z in conventional tandem mass spectrometers. First measurements using TIMS PASEF stepping on glycopeptide-enriched plasma samples already enabled identification of 2423 unique glycopeptides from 408 glycosylation sites of 187 proteins using prototype glycoproteomics software.

Conclusions

PASEF and prototype software enable comprehensive glycoproteomics.

P206

Feasibility of phosphoproteomics on leftover samples after RNA extraction with guanidinium thiocyanate

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Topic: Post-Translational Modifications

Introduction
In daily practice, different types of biomolecules are usually extracted for large-scale 'omics' analysis with tailored protocols. However, when sample material is limited, an all-in-one strategy is preferable. While lysis of cells and tissues with urea is the accepted standard for phosphoproteomic applications, DNA, RNA and proteins can be simultaneously extracted from small samples using acid guanidinium thiocyanate- phenol-chloroform (AGPC). Use of AGPC for mass spectrometry (MS)-based phosphoproteomics has been reported, but not benchmarked.

Methods
Using high-resolution tandem mass spectrometry, a TiOx phosphoproteomic workflow and recent bioinformatic tools such as inferred kinase activity (INKA) analysis and phosphosite signature enrichment analysis (PTM-SEA), we compared urea- with AGPC-based protein extraction, profiling phosphorylations in the DNA damage response pathway after ionizing irradiation of U2OS cells as proof of principle.

Results
On average we identified circa 9000 phosphosites per sample with both extraction methods. Moreover, we observed high similarity of phosphosite characteristics (e.g. 94% shared class 1 identifications) and deduced kinase activities (e.g. ATM, ATR, CHEK1/2, PRKDC).

Conclusions
AGPC-based sample extraction can replace standard cell lysates for phosphoproteomic workflows and may thus be an attractive way to obtain input material for multiple omics workflows, yielding several data types from a single sample.

P207

Investigating phosphopeptide isomers using data-independent and data-dependent mass spectrometry

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Topic: Post-Translational Modifications

Introduction
Data dependent acquisition (DDA) shotgun mass spectrometry has been used to identify and quantify thousands of phosphorylation sites. However, there are certain limitations of DDA, namely, the stochastic nature of precursor ion selection and the identification of co-eluting precursor ions. In particular, co-eluting phosphopeptide isomers may compromise the site-localization probabilities of phosphosites present on co-eluting isomers. Additionally, dynamic exclusion is hypothesized to potentially exclude closely eluting phosphopeptide isomers, affecting the number and consistency of identifications across runs. Data independent acquisition (DIA) is an alternative acquisition method where precursor ions are fragmented within predefined m/z windows over the chromatographic retention time. It has previously been shown to improve reproducibility and quantification in proteomic samples.

Methods
We investigated a dataset consisting of 10 biological replicates of nocadozole treated and untreated U2OS cells (PXD006056) (1). Each sample replicate was phosphopeptide enriched and measured using an AB Sciex 6600 TripleTOF mass spectrometer in DDA and SWATH-DIA mode separately. We compared the DDA results processed by MaxQuant and to the DIA results analysed by OpenSwath to investigate the identification and retention time attributes of phosphopeptide isomers in these samples.

Results
Within the phosphopeptides identified by both DDA and DIA methods, DIA shows superior quantitative reproducibility compared to DDA across the 10 biological replicates. Reproducibility is crucial as phosphosites can only be inferred by direct evidence from the phosphopeptide. Additionally, DIA identifies more phosphopeptide isomers in the nocadozole-treated condition compared to DDA, though results are comparable in the control condition. We found that retention time differences between phosphopeptide isomers in this dataset averaged around 80-180 seconds, which is longer than the average dynamic exclusion time period used in DDA.

Conclusions
DIA shows promise towards consistently identifying phosphopeptide isomers in biological samples. Advantages of DIA for site-localization on peptides should be rigorously investigated.

References
1. https://doi.org/10.1038/nbt.3908

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Role of posttranslational modification, citrullination in polarization to proinflammatory and anti-inflammatory macrophages

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Topic: Post-Translational Modifications

Introduction
Macrophages play a pivotal role in the development of low-grade chronic inflammation present in many civilization diseases. They can be classified according to two main phenotypes: “proinflammatory” M1 macrophages, which are responsible for the clearance of pathogens and “anti-inflammatory” M2 macrophages, that are involved in resolution of inflammation and tissue repair. In recent years, citrullination, an irreversible enzymatic posttranslational modification by which arginine is converted to citrulline by the Protein Arginine Deiminase (PAD) family of enzymes has gained much attention due to its involvement in various physiologic and pathologic conditions. The loss of positively charged arginine can alter protein structure and function, affects protein-protein interactions, leads to proteolysis as well as acts as auto-antigen.

Methods
The aim of our study was to investigate the role of PAD enzymes and its downstream effects, such as protein citrullination in polarization of macrophages to M1 and M2. We used human monocytic cell line THP-1 differentiated by PMA to macrophages. The cells were further differentiated to M1 and M2 macrophages by LPS and IL-4, respectively, and treated with PAD inhibitor – BB-Cl-amidine. Then we measured mRNA and protein expression of different PAD isoforms. To identify protein citrullination sites we applied mass spectrometry DIA method and hypercitrullinated library approach.

Results
PAD2 and PAD4 were the most abundant isoforms in THP-1 macrophages with PAD2 being downregulated in anti-inflammatory macrophages and PAD4 being upregulated in proinflammatory macrophages. Moreover, treatment with PAD inhibitor decreased expression of proinflammatory markers (IL-6, TNF-α) in M1 macrophages measured by ELISA and real-time PCR. Proteomic approach identified different patterns of protein citrullination in M1 and M2 macrophages.

Conclusions
Taken together our results indicate different expression of PAD enzymes as well as specific pattern of protein citrullination in proinflammatory and anti-inflammatory macrophages. The exact functional consequences of the revealed alterations require further investigations.

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Co-enrichment of hydroxylated and phosphorylated tyrosine: A new technique for studying the oxidated brain kinome.

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Topic: Post-Translational Modifications

Introduction
Parkinson's disease (PD) is a neurodegenerative disorder characterized by a loss of dopamine-producing neurons in the mid-brain. The disease has oxidative stress (OS) as a pathological feature which is possibly increased via the consumption of the therapeutic Levodopa (L-DOPA). L-DOPA is an amino acid that is the intermediary between tyrosine and the neurotransmitter; dopamine, the presence of L-DOPA can result in protein incorporation. The presence of L-DOPA has yet to be explored by proteomics in a clinical setting, as such this work aimed to generate an enrichment methodology for the study of L-DOPA map the location of L-DOPA in the human brain proteome.

Methods
Tryptic peptides of human neuroblastoma cell (SH-SY5Y) proteins containing C-14 L-DOPA were enriched using Aluminum resin and profiled for binding efficiencies using scintillation counting. Non-radioactive treatments were performed, and the resultant tryptic digest was enriched for L-DOPA with STAGE-tipping used for sample clean up. The resultant data were analyzed using PEAKS Studio X +®.

Results
Scintillation analysis revealed peptide enrichment resulting in a fraction containing 90% of the radioactive tracer. Analysis of the resultant cleaned peptides by PEAKS PTM searching revealed that the highest abundance modification in the enriched fraction was alternatively saturated with Phosphorylated peptides. The co-enrichment reveals the links between phosphorylation sites affected by the L-DOPA replacement and the potential disruption to the kinome.

Conclusion
This study has developed a method for the simultaneous enrichment of L-DOPA and phosphorylated peptides. This methodology can now be used to study clinical samples for the presence of L-DOPA in PD and as an OS marker, other neurological diseases. The co-enrichment provides context on the impacts on cell signalling.

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Oxidative PTMs in peptides – Understanding the physiological consequences of physical plasma

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Topic: Post-Translational Modifications

Introduction: Enzymatically induced post-translational modifications (PTMs) of proteins are important for many signaling pathways with potentially drastic changes in structure and activity of these molecules [1]. It is assumed, that similar chemical groups can be created by reactive oxygen or nitrogen species (RONS) such as H_2O_2, $^1O_2$ or ONOO– that are generated by physical plasma (oxidative PTMs). Creating a library of such oxidative PTMs facilitates global mapping of PTMs in mammalian tissue and finally foster understanding of complex redox controlled processes.

Methods: Here, a kINPen argon plasma source [2] and a COST helium plasma jet [3] running with different gas mixtures were used to simulate oxidative conditions on 10 different artificially designed model peptides in water and phosphate buffered saline (PBS). The modified peptides were analyzed by liquid chromatography coupled to high-resolution mass spectrometry and evaluated subsequently by a multi-PTM search bioinformatic workflow.

Results: It turns out, that the two plasma sources produce a completely different modification pattern based on different reactive species. Single oxidations and chlorinations occur more often after COST jet treatments, dioxidations or nitrations are more common with kINPen treatments.

Conclusions: The targeted oxidation of amino acids in proteins, e.g. by the plasma-generated redox signal molecule H_2O_2, or other reactive species lead in smaller amounts to an eustress redox signal and thus could activate signaling pathways for the wound healing process. In higher amounts, this species could cause distress oxidation of cancer cell-specific proteins and thus kill them. This work can give information about which possible modifications at which amino acids can be expected after a plasma treatment.

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P211

A rigorous and objective approach to validating individual peptide-spectrum matches (PSMs)

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Topic: Post-Translational Modifications

INTRODUCTION: LC-MS/MS technology is increasingly being used to identify native peptides (e.g., immunopeptidomics) and post-translational modifications (PTMs). In these applications, search spaces are often large and identifications must often be based on a single peptide-spectrum match (PSM), making it difficult to confidently establish the presence of a peptide of interest within a biological sample. This challenge has recently been highlighted by efforts to identify proteasomally-spliced peptides in the immunopeptidome. Currently, the gold standard for validating individual PSMs is comparison to synthetic validation peptides. These comparisons, however, can be fraught with subjectivity. We have developed a partially-automated approach--PSM Validation with Internal Standards (P-VIS)--that ensures greater rigor and objectivity when using synthetic peptides to validate PSMs.

METHODS: When comparing the fragmentation spectra of a peptide in a biological sample and a synthetic validation peptide, an investigator must subjectively determine what degree of similarity indicates a valid match. In the P-VIS approach, a set of synthetic internal standard peptides (ISPs) is spiked into both the biological sample and the synthetic peptide sample, providing a set of valid matches. A computer program, PSM_validator, then uses these valid pairs to assess if the similarity between the synthetic peptide spectrum and the biological peptide spectrum falls within the predicted range for a valid match. To benchmark this new approach, we carefully selected 22 synthetic peptides, analyzed them by LC-MS/MS, and tested the accuracy of P-VIS at distinguishing two peptides that are similar but different.

RESULTS: For benchmarking experiments, sensitivity (the percentage of valid matches that were determined valid) exceeded 80%, and specificity (the percentage of invalid matches that were determined invalid) was 100%.

CONCLUSIONS: P-VIS is an easily-implemented, systematic, and accurate approach to determining the validity of individual PSMs. Use of P-VIS can provide greater confidence in peptide and PTM identifications, facilitating various downstream biological applications.

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Deep Profiling of Tyrosine Phosphorylation in Gastric Cancer Cells

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Topic: Post-Translational Modifications

Protein phosphorylation is known to play a key role in both normal cellular physiology and to be frequently modified in disease states. As such, protein kinases are frequent therapeutic targets in the treatment of cancer and other diseases, and quantitative analysis of phosphorylation changes can be crucial to both mechanism of action studies and biomarker discovery. Cellular levels of tyrosine phosphorylation are significantly lower than serine and threonine and require specific immuno-enrichment for deep profiling. In this study, we combine peptide-level enrichment of phosphotyrosine with a TIMS enabled QTOF to both identify sites of tyrosine phosphorylation and measure changes in that phosphorylation upon treatment of a gastric cancer cell line with a specific tyrosine kinase inhibitor.

The gastric cancer cell line MKN-45 was treated with and without the c-Met inhibitor SU11274 (1 uM, 2h). Proteins were digested with trypsin and resultant peptides were immunoenriched using the anti-phosphotyrosine antibody P-Tyr-1000 (Cell Signaling Technology, Inc.). The resulting extracts were separated by nano HPLC (nanoElute, Bruker) on 250 mm x 75 µm, 1.6 µm (IonOpticks, Australia). 60 min gradients at 400nL/min were analyzed on a trapped ion mobility Q-TOF (timsTOF Pro, Bruker Daltonics) operating in PASEF mode. Data were processed in PEAKS X+ software (Bioinformatics Solutions Inc).

We have coupled this to a modified PASEF method on a trapped ion mobility QTOF which significantly improves peptide identification at low levels. The incorporation of the high- resolution ion mobility also allowed the resolution of coeluting, isobaric peptides that differ only the site of phosphorylation. Of the several thousand tyrosine phosphorylation sites identified in this study, several hundred were found to significantly decrease following treatment with the c-

Met inhibitor. These include sites on the target kinase, known downstream substrates, and potentially novel biomarkers of c-Met signaling.

P213

Identifying HDAC3 as a delactylase using quantitative proteomics

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Topic: Post-Translational Modifications

Introduction:
We have recently reported lysine lactylation (Kla) as a new type of histone post-translational modification (Nature, 574:575-580, 2019), which can be stimulated by the Warburg effect-derived lactate. This discovery offers a new opportunity to understand function of the Warburg effect and glycolysis in normal physiology as well as in diseases. Nevertheless, the key regulatory elements for this pathway, “erasers”, “writers”, and “readers”, remain unknown, representing a major bottleneck to study its biology. In this abstract, we report the identification of HDAC3 as a delactylase. We used quantitative mass spectrometry to identify its target sites on core histones.

Methods:
Delactylase screening assay was performed by incubating 0.5 µM of acid-extracted histones with a recombinant HDACs (0.08 µM of HDAC1-5) in the reaction buffer (HDAC1-5: 25 mM Tris pH 8, 130 mM NaCl, 3.0 mM KCl, 1 mM MgCl2 , 0.1% PEG8000, pH 8.0) for 16 hours at 37 degree. Histones were digested with trypsin and analyzed by HPLC-MS/MS with a gradient of 5% to 50% HPLC buffer B (0.1% formic acid in acetonitrile, v/v) in buffer A (0.1% formic acid in water, v/v).

Results:
Among 11 HDACs, HDAC3 has the strongest delactylase activity in vitro, and Class I HDAC inhibitor (TSA and Apicidin), diminished the activity. Stable Isotope Labeling with Amino acids in Cell culture (SILAC)-MS/MS quantification confirmed that HDAC3 exhibited significant delactylase activity and we identified multiple histone Kla sites regulated by HDAC3. Given that HDAC3 is a known deacetylase, kinetic studies will be carried out to measure and compare the Kcat and Km for delactylation versus deacetylation activities of HDAC3.

Conclusions:
Identification of HDAC3 as a delactylase enzyme and its target Kla sties on core histones by mass spectrometry.

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The predictive power of N-glycan structure profiling can stratify rheumatoid arthritis patient response to methotrexate for informing efficient treatment strategies

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Topic: Precision Medicine in Wellness and Disease

Introduction: Rheumatoid Arthritis (RA) is an autoimmune disorder, which causes systemic inflammation leading to increased morbidity and mortality. The first-line treatment is methotrexate (MTX), an inhibitor of folate biosynthesis. However, dosing is rarely optimized and, as a monotherapy, this results in an adequate clinical response for only one-third of patients. Treatment escalation is required for those that do not respond sufficiently, and commonly includes expensive biopharmaceuticals.

Predictive biomarkers are urgently needed to improve RA treatment efficiency. Specifically, markers that predict response to MTX will allow for earlier intervention with the most appropriate strategy, informing whether MTX dosing strategy requires optimization or whether an alternative therapy is required. Circulating protein glycosylation has been previously reported in RA plasma and specific motifs have been found to correlate with disease severity and progression.

Methods: N-glycan structures were quantified by a PGC-LC-ESI-MS method applied to plasma from a cohort of 40 individuals (n=60 samples): 20 controls and 20 RA patients. For all RA patients, samples were collected pre-treatment and after 16 weeks of MTX therapy. Of the RA patients, 10 were classified as good responders and 10 were classified as poor responders based on the change in 28-joint Disease Activity Score.

Results: Hierarchical clustering distinguished patients with different MTX responses using the glycan profiles at baseline (pre-treatment). Unblinded analysis revealed 23/45 structures significantly differed ($p<0.01$) between non-RA and RA patients, with many structures consistent with reported analyses of serum-sourced IgG. Uniquely, we observed 5/45 of structures as significantly different between good and poor responders at baseline, supporting the hypothesis that the plasma glycome is a rich source of predictive biomarkers for improving treatment strategies for RA.

Conclusions: These data suggest that specific structures within the plasma N-glycome can be predictive of RA patient response to MTX treatment, allowing discriminative analysis of pre-treatment cohorts.

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An Ultra High-throughput Plasma Protein Profiling (uHTPPP) Workflow Using the New Orbitrap Exploris 240 Mass Spectrometer

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Topic: Precision Medicine in Wellness and Disease

Introduction:
Analyzing biomarkers from blood is ideal for early disease detection and monitoring therapeutic response. However, proteomics analysis of blood remains challenging because of high dynamic range of plasma proteome and individual heterogeneity. Conventional proteomics workflows, based on manual manipulation and nanoflow, excel in sensitivity but lack throughput to analyze population heterogeneity. We developed a standardized plasma profiling workflow solution (uHTPPP) using the new Orbitrap Exploris 240 Mass Spectrometer. This workflow enables automated sample preparation and rapid LC separation, while the Orbitrap Exploris 240 delivers robust performance and high quality data for large scale cohort studies.

Methods:
Top 14 abundant proteins were depleted from pooled human cancer serum, and then processed by automating the Thermo Scientific™ EasyPep™ 96 well MS Sample Prep Kit on a Hamilton STARLet liquid handling system with a high-throughput filtration method [MPE]2 — Monitored Multi-flow, Positive Pressure Evaporative Extraction module. Digested peptides (200-500 ng) were loaded onto EvoTip disposable trap columns and separated by the Evosep One LC system at the throughput of 60 samples a day. Resulting MS2 spectra were annotated by spectral library matching using Thermo Scientific™ Proteome Discoverer™ 2.5 with feature matching and MS1 quantification.

Results and Conclusions:
We showed that the new Orbitrap Exploris 240 delivers good run-to-run reproducibility while maintaining instrument stability for a long period of time. Less than 5%CV of protein identification was obtained from system suitability runs before and after 150 injections. 400-500 plasma proteins were identified covering >5 orders of dynamic range using feature matching. More than 60% of proteins were quantified with<20% CV. Finally, a standardized search template was created using a PROSIT spectral library search with LFQ quantification. In conclusion, the uHTPPP workflow shows robust MS performance and demonstrates increased throughput for analyzing large cohorts by at least 10-fold compared to conventional nanoflow methods

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Evidence of involvement of the Renin-Angiotensin System in Chronic Lung Allograft Dysfunction

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Topic: Precision Medicine in Wellness and Disease

Introduction

Chronic lung allograft dysfunction (CLAD) is the major cause of death following lung transplantation. Angiotensin II (AngII), the main effector of the renin-angiotensin system, elicits fibrosis in both kidney and lung. We identified 6 AngII-regulated proteins (RHOB, BST1, LYPA1, GLNA, TSP1, LAMB1) increased in urine of patients with kidney allograft fibrosis. We hypothesized that renin-angiotensin system is active in CLAD and that AngII-regulated proteins are increased in bronchoalveolar lavage (BAL) of CLAD patients.

Methods

We performed immunostaining of AngII receptors (AGTR1 and AGTR2) and TSP1/GLNA in 10 CLAD lungs and 5 controls. Following the development and optimization of parallel reaction monitoring (PRM) assays, we quantified proteotypic peptides corresponding to AngII-regulated proteins in BAL of 40 lung transplant recipients (CLAD, stable and acute lung allograft dysfunction (ALAD)). Classifiers based on combined peptide concentrations were built using machine learning algorithms and their diagnostic accuracy and predictive value were assessed in discriminating or predicting CLAD.

Results

Immunostaining demonstrated significantly more AGTR1+ cells in CLAD vs control lungs ($p=0.02$). TSP1 and GLNA protein expression positively correlated with the degree of lung fibrosis ($R2=0.42$ and 0.46, respectively). PRM analysis showed a trend towards higher concentrations of AngII-regulated peptides in BAL of patients with CLAD at the time of bronchoscopy, and significantly higher concentrations of BST1, GLNA and RHOB peptides in patients that developed CLAD at follow-up ($p<0.05$).) Peptide concentrations correlated most significantly with the degree of neutrophilic inflammation and CMV viremia, and less significantly with A-grade rejection. Support vector machine classifier discriminated CLAD from stable and ALAD patients at the time of bronchoscopy with AUC 0.86, and accurately predicted subsequent CLAD development (AUC 0.97).

Conclusions

Our study suggests that renin-angiotensin system is active in CLAD. AngII-regulated peptides measured in BAL may accurately identify patients with CLAD and predict subsequent CLAD development.

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Deciphering tumoral biology by novel nano-functional proteomics approaches: Novel therapeutic targets in chronic lymphocytic leukemia & leptomeningeal diseases.

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Topic: Precision Medicine in Wellness and Disease

In post-genome era having sequenced the human proteome, one of the most important pursuits is to understand the function of gene-expressed proteins. Despite immense progress in molecular biology and genetics, only a small fraction of the proteome is understood at the biochemical level. Systems Biology and proteomics strive to created detailed predictive models for molecular pathways based upon the quantitative behaviour of proteins. Understanding these dynamic networks provides clues into the consequence of aberrant interactions and why they lead to disease like cancer. However, collecting biochemical data about protein behaviour at scale has been daunting. Historically, methods capable of collecting quantitative data on biochemical interactions could only be used for one or a few proteins at the time. Here, we show the combination of two technologies that together could lead to the ability to measure binding events in real time for many protein interactions simultaneously using a label-free technology. This could revolutionize the study of protein interactions networks by enabling quantitative comparisons of binding affinities across many molecular species, as well determining the kinetics rates of binding and release. Here, we describe the approach and the application in the idenfication of novel nanomedicine targets on chronic lymphocytic leukemia and leptomeningeal disease.

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LC-MS/MS analysis of the human tear proteome

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Topic: Precision Medicine in Wellness and Disease

Introduction: Eye diseases are widespread in the population, and can cause a multitude of side effects, such as discomfort, dryness, visual disturbance and potential damage to the ocular surface, with a significant reduction in quality of life. Proteins in tears have an important role in eye health, and have been shown previously to correlate with some diseases. The comparative analysis of proteins from tears could identify biomarker proteins for many ophthalmological diseases. We are currently developing a robust and sensitive method for profiling tear proteins, from healthy volunteers.

Methods: Tear samples were collected on tear strips and reductive alkylation followed by tryptic digestion was performed. Different LC-MS/MS methods have been compared including microflow and regular flow HPLC as well as incorporating pre-fractionation of peptides to increase proteomic coverage. LC-HRMS/MS analysis on a QqTOF (Sciex TripleTOF 5600+) system was performed followed by database searching using Protein Pilot (Sciex) and Scaffold-Q+ (v4.9.0, Proteome Software) for sample and workflow comparisons. We are currently compiling a database of all proteins found in tears from healthy volunteers. Targeted analyses via SWATH acquisition and MRM analyses are being compared for quantitative analysis of these proteins.

Results: We have detected over 700 proteins in tears, with 1% FDR protein threshold. Sample pre-fractionation by reverse-phase HPLC and by mixed mode ion exchange solid-phase extraction were both useful in increasing proteome coverage substantially. Sample preparation has also been optimized including protein extraction from the tear strips to digest volume and conditions. Normal variations in protein distribution from several healthy volunteers is being investigated to better ascertain which proteins may be relevant candidates as biomarkers of specific eye diseases.

Conclusions: Once a targeted method is established, the optimized method will be applied for studying several eye related pathologies, including certain rare eye diseases and corneal transplant rejection.

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Ex vivo glucocorticoid-induced secreted proteome approach for discovery of glucocorticoid-responsive proteins in human serum

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Topic: Precision Medicine in Wellness and Disease

Introduction: Glucocorticoids which are used in pharmacological doses for a variety of medical conditions, and endogenous glucocorticoid excess – Cushing's syndrome, may result in several adverse effects, but currently there is no clinically useful biomarker of glucocorticoid activity. The aim of this study was to identify glucocorticoid-responsive proteins potentially measurable in human serum that might have clinical utility as biomarkers, for use in therapeutic drug monitoring and the diagnosis of cortisol excess or deficiency.

Methods: A three-phase protein biomarker discovery strategy was used. Proteomic biomarker discovery and qualification was conducted on the secretome of ex vivo-stimulated peripheral blood mononuclear cells (PBMC) isolated from 6 volunteers, incubated ± dexamethasone 100 ng/mL for 4h and 24h. Untargeted proteomics with label-free quantification was conducted to discover candidate proteins which were quantified using targeted proteomics by a custom multiple reaction monitoring mass spectrometry (MRM-MS) assay. Finally, 5 candidate circulating biomarkers were measured by immunoassay in serum samples from an independent clinical cohort (n=20), sampled at 1200h before and after a single dose of 4 mg oral dexamethasone administered at midnight.

Results: Paired analysis of the discovery secretome proteomics data (576 and 280 proteins for the 4h and 24h secretomes, respectively) generated a shortlist of 45 candidates, with 43 measured in the final MRM-MS assay, 16 of which had significant differential expression in at least one time point. In the validation cohort examining serum protein concentrations, 3/5 proteins were dexamethasone-responsive, two significantly decreased: lysozyme C ($P<0.0001$); nucleophosmin-1 ($P<0.01$), while high mobility group box 2 significantly increased ($P<0.01$).

Conclusions: Using an ex vivo proteomic approach in PBMC, we have identified and verified circulating glucocorticoid-responsive proteins which may have potential as serum biomarkers of glucocorticoid activity.

P220

Precision Medicine in Human Transplantation: matching immunogenic epitopes on HLA proteins to reduce rejection risk and prolong survival

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Topic: Precision Medicine in Wellness and Disease

Introduction

Human leukocyte antigens (HLA) are cardinal immunogenic proteins causing transplant rejection, but the enormous heterogeneity (>25,000 alleles) of HLA genes makes donor-recipient matching challenging. To overcome this, we combined next-generation sequencing, structural biology, and computational medicine to define the sequence, conformation, and distribution of critical B-cell epitopes on HLA proteins in the context of allorecognition to be used in an innovative matching method to improve transplant outcomes.

Methods

1846 kidney patients/donors were sequenced at 11 HLA genes, and genotypes were converted to epitopes in silico using HLAMatchmaker algorithms. Allele and epitope frequencies were compared across ethnically diverse donor and patient populations. Computational organ allocation models were developed using ABO-matching and epitope-matching scores. Simulations were run in R, using sample sizes consistent with annual Canadian recipient and donor rates.

Results

We identified 150 antibody-defined epitopes within the population. Each HLA protein expressed 0–17 epitopes, predominantly located in the T-cell peptide-binding groove. While the majority of alleles occurred at low frequencies (>50% present in <1% of subjects), epitopes were more broadly and uniformly distributed (>75% present in >30% of subjects). Most eplets were gene restricted (occurring in multiple alleles of the same gene) while a small number showed inter-locus distribution (occurring on alleles of different genes), particularly across class I genes. Simulation modeling showed epitope-matching markedly reduced HLA mismatch by >60% with the median mismatch score declining from 27.35 to 9.3 across all genes, and a high (almost 100%) probability of identity at the cardinal class II antigens. This result was possible in single transplant units, avoiding the need for national organ sharing.

Conclusions

Matching for immunogenic epitopes dramatically reduces HLA heterogeneity and improves the opportunity for donor-recipient compatibility. Canada is working to adopt this strategy in a unique program of epitope-based matching to reduce organ rejection and prolong graft survival.

P221

Sample Size Requirements for Meaningful Interpretation of Mass Spectrometry-Based Proteomic Analysis of Human Vitreous

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Topic: Precision Medicine in Wellness and Disease

Introduction: Prior studies have shown that proteomic analysis of vitreous can identify pathways with critical roles in retinal disease. However, the reliability of these results is currently unknown, as power analyses have not previously been calculated. The goal of this study was to complete a power analysis for vitreous proteomic studies to facilitate confident identification of novel disease pathways.

Methods: Undiluted vitreous was obtained from patients undergoing vitrectomy for macular hole/epiretinal membrane repair (controls, n=25) or proliferative diabetic retinopathy (PDR, n=23). Both groups were treated individually to define biological variability. Pooled samples were combined and aliquoted to capture multiple aspects of technical variation. Samples were analyzed using TMT-labeling and ESI-MS/MS. In total, five 10plex kits were run. This resulted in a single data matrix comprised of data from each 10plex. This study was approved by the affiliated IRBs and adhered to the tenets of the Declaration of Helsinki.

Results: Across all samples, the total number of unique proteins was 1,152 in experiment 1 and 1,191 in experiment 2. The average coefficient of variation (CV) across patients was 36.9%. The average CV across technical replicates was 4.2%. This suggests biological variability accounted for the majority of variability in this dataset. Power analysis revealed that 10 samples are required to achieve a power level of 0.90 for proteins with log2 fold change of at least 1.194. Differential expression analysis demonstrated significant upregulation of metabolic pathways known to be dysregulated in diabetes in PDR samples.

Conclusions: Quantitative MS analysis of vitreous is a reliable method to determine proteins and pathways involved in retinal disease. The required sample size will vary depending on the disease and effect size, but, in general, a sample size of 10 is sufficient to generate robust and meaningful data.

P222

Extending cross-linking analyses with XlinkX/PD to low FDR-rates and structural interpretation

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Topic: Protein Structures and Complexes

Introduction – Crosslinking mass-spectrometry (XL-MS) has become a powerful structural biology application for protein structure determination and protein interaction mapping. With our analysis software pipeline XlinkX we bridge this highly specialized technique to non-expert laboratories worldwide on the easy-to-use and error tolerant Proteome Discoverer platform. Advancing insights into controlling FDR levels, filtering false positives, and connecting to the structural context require constant improvements. Here we present extensions to XlinkX/PD that result in low false positive rates and enabling easy export of the results for structure mapping.

Methods – We used existing datasets to test the performance of the improvements to XlinkX/PD. All data was analyzed with Proteome Discoverer 2.5.

Results – Our improved FDR control regime, operating at the identification level of individual fragmentation scans, combines fragmentation scans and corresponding cross-links automatically and results in FDR rates close to the expected thresholds (without any score cutoff). At the fragmentation scan level we detected, using multiple data sets of different complexity, for a target FDR of 1% a real FDR of 1.5% for CSMs and 2.6% at the cross-link level respectively, which is well within statistical variation. Moreover, we integrated a blastp supported link to pyMol that allows for an automated mapping of the detected cross-links onto PDB structures under consideration. Finally, an improved export to mzIdentML v1.2 supports submission of results to repositories like PRIDE and jPost, which enables optimal transparency to other researchers.

Conclusions – With the added functionality to XlinkX/PD we made improvements to the FDR control that bring the results in line with the expected values. Additionally, by integrating pymol export functionality to PDB structures and research data repositories we enable non-expert laboratories to take advantage of this powerful analysis technique.

P223

Hsp40 Affinity Identifies Proteins Destabilized by Cellular Arsenite Treatment

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Topic: Protein Structures and Complexes

Introduction: Environmental oxidants and electrophiles can damage proteins. Although toxins, such as arsenite, can activate misfolded protein stress responses, identifying the individual proteins that are misfolded is challenging. Hsp40 co-chaperones bind and recruit misfolded proteins to the hub Hsp70 chaperoning cycle. As such, we considered that Hsp40 proteins might serve as recognition elements to identify proteins that misfold in response to cellular exposure towards oxidizing metals.

Methods: We evaluated and optimized several human Hsp40 proteins for their ability to recover cellular clients through affinity purification coupled with quantitative mass spectrometry using Tandem Mass Tags (TMT-AP-MS). We overexpressed J-domain inactivated DNAJB8 in HEK293T cells, briefly (15 min.) treated the cells with arsenite ($NaAsO_2$) or $CdCl_2$, and performed TMT-AP-MS. Destabilization of protein targets was orthogonally validated by limited proteolysis combined with parallel reaction monitoring.

Results: We find that DNAJB8 reproducibly and specifically binds and co-immunoprecipitates hundreds of proteins even in the absence of crosslinking. This subproteome is relatively destabilized compared to the general proteome. Cellular arsenite treatment increases DNAJB8 association to several proteins including TDP43, RNA-binding proteins, and the pyruvate dehydrogenase subunit PDHA1. Limited proteolysis analysis validated that most of these proteins are indeed destabilized by brief arsenite treatment. Cd treatment destabilizes PDHA1 as well, but no other arsenite targets, indicating that protein destabilization is metal-specific.

Conclusions: Hsp40 affinity is an effective way to identify proteins that are destabilized due to toxicant exposure.

P224

Not all roads lead GAA to lysosomes

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Topic: Protein Structures and Complexes

Alpha Acid Glucosidase (GAA) is a lysosomal enzyme essential for the degradation of glycogen into glucose in lysosomes; mutations in gene encoding for this enzyme are causative of a form of Lysosomal Storage Disease known as Pompe Disease (PD). Although the biochemistry GAA activity and genetic basis of the associated disorder are well characterized, the routes followed by wild type GAA to reach lysosomes and impaired in presence of mutants are still unclear. In order to elucidate the fate of recombinant protein once got into the cell the intracellular pathways controlling GAA protein traffic in physiological and pathological conditions, as well as the internalization process of rhGAA protein was investigated by the analysis of GAA's interactome. The identification of intracellular factors regulating the traffic of GAA protein, and affected in PD, might unravel the cell processes impaired in presence of GAA mutant causative of this disorder. Proteomics results have showed that, in physiological condition, endogenous GAA interacts with different cytoskeleton proteins and in particular, the interactions are isoforms specific, suggesting that they might occur at different step during the traffic of GAA from endoplasmic reticulum to lysosomes. Gelsolin, a validated GAA protein partner, has a key role in the early stages of GAA trafficking resulting a potential target for definition of new therapeutic strategy in PD.

P225

MM500: Unraveling the Proteomic Landscape of Malignant Melanoma

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Topic: Proteogenomics

Introduction: Melanoma of the skin is the sixth most common type of cancer in Europe and accounts for 3.4% of all diagnosed cancers. More alarming is the degree of recurrence that occurs with approximately 20% of patients lethally relapsing following treatment. To gain a full understanding of melanoma biology requires substantial information about the proteome through the course of disease. Here we present the MM500 meta-study, an initiative to provide a comprehensive proteomics landscape of melanoma from the analysis of 500 patient tumor samples. To accomplish this goal we applied robust clinical proteomic platform that encompasses automated biobanking of patient samples, tissue sectioning, histological examination, efficient sample preparation and MS protocols.

Methods: Tumor samples were histopathologically characterized and analyzed using established proteomic workflows. Samples were analyzed in several LC-MS/MS systems; including EASY-nLC 1000 and Ultimate 3000 (Thermo Fisher Scientific) coupled to Q Exactive Plus and Q Exactive HFX Orbitrap instruments (Thermo Fisher Scientific), respectively. MS raw files were analyzed using Proteome Discoverer and/or Spectronaut softwares. Bioinformatics analyses were performed using Perseus software.

Results: Through global proteomics, phosphoproteomic and acetylomics we unraveled a melanoma proteome comprising 13,456 protein-coding-genes which covers more than 75% and 65% of the melanoma transcriptome and the human proteome, respectively. This was based on the identification of more than 346000 non-redundant peptides, 53000 phosphopeptides and 4400 lysine acetylated peptides. We annotated the cellular compartment and human chromosome localizations of identified proteins, and outlined a map of melanoma protein rank abundance were the expression of melanoma driver gene mutations were positioned head-to-head with the corresponding proteins.

Conclusions: Mapping melanoma expressed proteins with quantitative, spatio-temporal localization, mutations-, isoforms- and PTMs-variants will aid in understanding the disease mechanisms on a molecular level along the axis of gene expression.

P226

SIRT7 as a Novel Regulator of Cellular Senescence

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Topic: Proteogenomics

Introduction: The sirtuins are a family of NAD+-dependent protein deacetylases that have been implicated in senescence. Cellular senescence is the process where a cell limits its proliferative ability and ceases to divide. This process also triggers the senescence-associated secretory phenotype (SASP) where cells secrete high levels of inflammatory cytokines and growth factors. Eliciting this immune response via SASP secretion promotes the clearance of immune cells, a process known as senescence surveillance. Oncogene induced senescence (OIS) facilitates senescence surveillance and the removal of senescent cells.

Methods: SIRT7 was immunoprecipitated from primary human lung fibroblasts (IMR90) in exogenous overexpression and endogenous conditions. Mass spectrometry was utilized to determine protein interactors during senescence, with an emphasis on E3 ubiquitin ligases.

Results: We discovered that the least studied sirtuin, SIRT7, which has histone and non-histone substates, is targeted for degradation upon oncogene-induced senescence (OIS) and this precedes the upregulation of senescence associated proteins like p16 and Lamin B1. Remarkably, rescue of SIRT7 loss during OIS by exogenous SIRT7 expression delayed senescence and importantly, diminished expression of SASP. These data suggest SIRT7 is a key regulator during senescence. Through immunoprecipitation-mass spectrometry (IP-MS), we determined E3 ubiquitin ligases that interact with SIRT7 upon senescence induction.

Conclusions: Immunoprecipitation mass spectrometry facilitated the discovery of a ubiquitin ligase that regulates SIRT7 degradation during senescence. This work provides novel insight into the regulation of senescence and potential targets for therapeutics of SASP regulators.

P227

Sarcoma proteogenomic using multi-layer omics profiles and patient-derived cancer models

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Topic: Proteogenomics

Introduction
Sarcomas are rare mesenchymal malignancies, consisting of more than 50 diverse histological subtypes and complex molecular backgrounds. To understand the molecular mechanisms underlying disease progression, and discover innovative medical seeds, we conduct functional proteogenomics. In functional proteogenomics, the comprehensive data are generated from genome to proteome, and the function study is achieved by the patient-derived cancer models such as cell lines, organoids, and xenografts.

Methods
To establish the patient-derived cancer models, we obtained the tumor tissues from patients who provided informed consent. The drug sensitivity was assessed in the established patient-derived cancer models using a library of anti-cancer drugs. Using surgically resected tumor tissues of more than 300 sarcoma cases, we tried to establish patient-derived cancer models, and used for the following analysis. Whole exome sequence data, and mutation and amplification of cancer-associated genes were obtained by next-generation sequence (NGS). Profiles of expression and kinase activity were obtained by mass spectrometry, and kinase substrate peptide arrays, respectively. Sample specific virtual proteome data were generated by NGS data and our original software, and used for mass spectrometric protein identification.

Results
We established 53 cell lines and 40 xenografts from tumor tissues of 24 histologically different sarcomas. NGS detected the mutation of drug target genes in multiple cell lines, xenografts, and tumors, while the results of mutation analysis were not concordant with the data of drug response assay. Mass spectrometry analysis detected the proteins which contained the sample specific mutations, and the proteins by which the samples were grouped according to the malignant features of tumors. Kinase activity was not consistent with the expression level in multiple kinases.

Conclusions
We generated a huge data set of patient-derived cancer models, and the integration of these data will generate novel innovative seeds for clinical applications in sarcomas.

P228

OncoProGx: Proteogenomics tool to generate theoretical sample-specific proteome database

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Topic: Proteogenomics

[Introduction] Global genomic aberrations cause malignant transformation and disease progression, and the diverse aberrant genetic mutations determine the characters of tumors and symptoms of patients with cancers. Proteome is a functional translation of genomic contents, and the proteome analysis should be based on the data of genomic aberrations of individual tumors. However, mass spectrometric protein identification relies on the public protein database such as Swiss-Prot, and the peptides translated from mutations unique to certain patient groups are not observed by mass spectrometry. This study aimed to develop a software to translate individual genome data to proteome data, so that the peptides with sample-specific mutations can be observed by mass spectrometry.

[Methods] We developed a novel proteogenomic tool, and designated it as OncoProGx. OncoProGx allows us to construct sample-specific proteome database for mass spectrometric protein identification. OncoProGx uses the data of whole exome, whole genome, and whole transcriptome. OncoProGx supports the identification of novel proteins, resulting from single nucleotide variants, splice variants and fusion gene. Combined with cRAP database, OncoProGx enables us to remove proteins commonly found in proteomics experiments that are present either by accident or through unavoidable contamination of protein samples. OncoProGx is equipped with graphic user interface. The performance of OncoProGx was evaluated using the data of whole exome sequencing, RNA sequencing, and LC-MS/MS of two pairs of patient-derived sarcoma cell lines and tumor tissues.

[Results] OncoProGx generated FAST format files, which are compatible with MasCot database search. The database created by OncoProGx had cancer-associated proteins, which included the peptides unique to patient-derived sarcoma cell lines and not recorded in SwissProt.

[Conclusions] OncoProGx enables the investigation of cancer-associated proteomic alterations. Peptides reflecting sample-specific mutations have a great potential for sarcoma research.

P229

Integration of comprehensive kinase activity assay and kinase inhibitor screen in patient-derived cancer cells for sarcoma proteogenomics

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Topic: Proteogenomics

Introduction: Sarcomas are mesenchymal malignant neoplasms that account for less than 1% of total malignancies, and consist of more than 50 different histological subtypes with different clinical behaviors and responses to treatment. The aberrant tyrosine kinase plays a key role in carcinogenesis and disease progression in sarcomas, and the tyrosine kinase inhibitors (TKIs) have been developed for treatments. Although the genetic mutation causes the aberrant kinase activities, it is not always predictive, and the mutation-to-phenotype relationship remains to be clarified. We aimed to interrogate the relation among genetic mutations, tyrosine kinase activities, and the responses to TKIs in sarcomas.

Methods: Patient-derived cancer cell lines were established using surgically resected sarcoma tissues. The mutation of tyrosine kinases was detected by the focused next-generation sequencing (NGS). The activity of 100 tyrosine kinases was examined using PamStation12. The anti-proliferative effects of TKIs were assessed in the established sarcoma cell lines.

Result: We established 53 patient-derived sarcoma cell lines, and characterized their molecular backgrounds, such as the presence of fusion genes or variable genomic contents, as well as the malignant phenotypes, such as the capability of spheroid formation and invasion. The focused NGS revealed the presence of mutations in the multiple tyrosine kinases in the sarcoma cells. The global in vitro kinase activity assay revealed the high activity of tyrosine kinases in the original tumor tissues and the established cell lines. The screen of TKIs resulted in the identification of multiple TKIs, which suppressed the proliferation of sarcoma cells at the considerably low level.

Conclusions: We generated the comprehensive omics data from DNA sequence to kinase activity, and the TKI response data in our original patient-derived sarcoma cell lines. Integration of those comprehensive multi-layer data will lead a novel indication of TKIs and predictive biomarkers in sarcomas.

P230

Proteogenomics for novel prognostic biomarkers in malignant peripheral nerve sheath tumor

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Topic: Proteogenomics

Introduction: Malignant peripheral sheath nerve tumors (MPNSTs) are malignant spindle cell sarcomas often arising from peripheral nerve. MPNSTs are biologically aggressive tumors with frequent local recurrence and distant metastasis, and refractory to chemotherapy and radiotherapy. In half cases, MPNSTs occur in the patients with neurofibromatosis type 1 (NF1), and NF1-associated MPNSTs have a worse prognosis than sporadic MPNST. By stratifying patients based on the malignant potentials, we can improve the clinical outcome of the patients using the optimized therapeutic approach. In this study, we aimed to develop the biomarker which reflects the malignant features of MPNSTs by proteogenomic approach.

Methods: Tumor tissues were obtained from 41 patients who were treated at the National Cancer Hospital (Japan) between June 2004 and March 2019. We established patient-derived cell lines and xenografts. We analyzed the protein expression of these tumors by using LC-MS/MS, and calculated emPAI via SwissPlot. Obtained emPAI values were statistically analyzed to identify the proteins associated with NF1, local recurrence, or distant metastasis. Focused next-generation sequencing (NGS) was achieved for cancer-associated genes using NCC Oncopanel.

Results: We obtained protein expression profiles consisting up to 5000. Among them, we identified proteins associated with NF1, local recurrence, and distant metastasis. Those proteins were known to be involved with poor prognosis in other malignancies, affecting the molecular pathways, and they are potentially biomarker candidates. Focused NGS identified mutations in cancer-associated genes.

Conclusions: We identified multiple proteins which could be candidates as biomarkers in MPNSTs. Their clinical significance should be validated using a simple method in the additional MPNST cases. Functional study using cell lines and xenografts, as well as the integration of proteome data with NGS mutation data will be our next challenge.

P231

Functional proteogenomics of patient-derived cancer model for sarcoma research

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Topic: Proteogenomics

Introduction
Sarcoma is a rare mesenchymal malignancy, characterized by the complex clinical and molecular features, accounting for less than 1% of all malignancies. To reveal the complex relationship between genotype and phenotype of sarcomas, and discover the novel innovative medical seeds, we conducted proteogenomics analysis.

Methods
We established well-characterized and clinically-annotated patient-derived sarcoma models using surgically resected tumor tissues. The anti-tumor effects of approved 210 anti-cancer drugs were examined using cell lines. Proteogenome data were generated by next-generation sequencing (NGS), and mass spectrometry.

Results
We established 53 cell lines and 40 PDXs of sarcomas of 24 histological subtypes, which included extremely rare types, such as CIC-rearranged sarcomas, and alveolar soft part sarcoma. These sarcomas were characterized by unique fusion genes such as CIC-DUX4 and ASPSCR1-TFE3, respectively. Because of their extreme rarity, no models are publicly available for these sarcomas. We confirmed that the established cell lines had capabilities to form organoids/spheroids. The screen of anti-cancer drugs identified multiple drugs, which demonstrated the remarkable anti-proliferative effects on the established cell lines. Those included the inhibitors for tyrosine kinases, proteasome, and histone deacetylase. NGS revealed the presence of mutations in the druggable genes. However, the presence of druggable mutations did not always link to the favorable response to molecular targeted drugs for those mutations. Interestingly, the response to anti-cancer drugs for certain molecular families or pathways was different between the 2D and 3D cultured sarcoma cell lines. The data of mass spectrometry and tyrosine kinase activity were integrated to identify the proteins associated with the response to drug treatments.

Conclusions
The integration of genotype and phenotype are ongoing to understand the molecular features of sarcomas. The discordance between the data of drug sensitivity and mutations will lead novel predictive biomarkers and indication of anti-cancer drugs in sarcomas.

P232

Calculating Sample Size Requirements for Single Cell Proteomics

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Topic: Single Cell Proteomics

Introduction
Understanding proteome dynamics is critical for understanding disease progression and treatment response. Single cell proteomics offers critical insight to these processes, yet logistical limitations restrict the number of cells that can be characterized. Thus, researchers need a method to determine how many cells are sufficient to identify statistically significant variation despite cell-to-cell heterogeneity.

Methods
We simulate time course data to explore the effects of biological variability and protein expression fold change on our ability to accurately detect protein abundance changes. Protein abundance trajectories were interpolated from subsamples of 7, 16, 20, 30, or 100 cells drawn from a much larger population created with a specified fold change and variability. A subsample was classified as accurately predicting the protein expression trend if its trajectory more closely matched the full population then it did to a null model.

Results
We determined the impact of fold change, variability, and sample size on accuracy in determining protein trends. Our method shows the false positive rate (proteins being classified as changing when they're not) and the false negative rate (proteins being classified as not changing when they are) that can be expected for specific parameterizations of fold change, variability and cell count. We also identify the expected number of proteins for specific fold change and variability parameters.

Conclusions
Our results serve as a practical guide for estimating the needed sample sizes to accurately detect results in single cell proteomics time series experiments.

P233

Deep proteome coverage and label-free quantitation analysis of limited numbers of human cells with a quadrupole-ion trap-Orbitrap mass spectrometer

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Topic: Single Cell Proteomics

Introduction
Mass spectrometry-based proteomic experiments utilizing samples derived from a small number of cells have great potential for answering biological questions but are accompanied by challenges. Here, we optimized the in-tube sample preparation method to test the proteome coverage and label-free quantitation performance of a Thermo Scientific Orbitrap Fusion Lumos instrument with limited numbers of Hela cells.

Methods
2.5 × 105 Hela cells were lysed in 500 µL 0.1% RapiGest, 5mM DTT in 50mM ammonium bicarbonate. Solutions equivalent to different number of cells (800, 400, 200, 80 and 40 cells) were subsequently prepared by diluting the lysate. Lys-C Trypsin digestion was employed. Peptides were loaded to a 50µm id C18 column for 90min gradient LC-MS analysis. MS2 HCD ion trap scan was used for peptide identification and high resolution MS1 was used for label free quantitation. Data were processed with Proteome Discoverer software with FDR set to 0.01.

Results
RapiGest lysis buffer, lower reaction volume and higher enzyme-protein ratio could achieve higher protein and peptide IDs. Around 2000 proteins and 6000 peptides were identified in 40 cells using the optimized method. And around 4300 proteins and 25000 peptides were identified in 800 cells. Median CVs of protein and peptide abundances were less than 20% and 11% in lower and higher loading amount samples, respectively, and the dynamic range could reach to 5 orders of magnitudes in both lower and higher loading amount samples.

Conclusions
The Orbitrap Fusion Lumos instrument showed deep proteome coverage and good label free quantitative performance for limited numbers of Hela cells (down to 40 cells) without using complicated microfluid-based sample preparation methods.

P234

Fully automated sample processing and analysis workflow for low-input label-free proteome profiling

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Topic: Single Cell Proteomics

Introduction: Deep proteome profiling of sub microgram and nanogram samples is still a technical challenge. The nanoPOTS platform1 has greatly improved the sensitivity of nanogram and single-cell proteomics, but dissemination to other laboratories has been hindered by its cost and complexity. Here we report a fully automated processing and analysis platform for low-nanogram proteomics and extending to single cells that uses only commercially available parts. Termed autoPOTS, this platform should be readily implemented in any proteomics laboratory.

Methods: Fluorescence-activated cell sorting was used to collect cells in a 384-well microplate with optical bottom. A low-cost Opentrons liquid handling robot with temperature control module was used for one-pot sample processing in a total volume of ~6 µL. An UltiMate WPS-3000 autosampler was modified with a 10-port valve for sample desalting and injection. The workflow was then evaluated using in-house-prepared 30-µm-i.d. nanoLC columns and Orbitrap Exploris 480 mass spectrometer. MaxQuant was used for data analysis.

Results: Pipetting performance and evaporation rates at different temperatures were evaluated. The sample processing workflow was programed to periodically dispense buffer or water to compensate for limited evaporation through the well plate sealing mat during incubation. The workflow was evaluated by analyzing 1–500 HeLa cells. An average of 166–~3300 protein groups were identified, with peptide coverage reduced by a relatively modest 24% for single cells and 12% for 150 cells relative to nanoPOTS. We used autoPOTS to profile ~1200 proteins from ~130 B and ~130 T lymphocytes isolated from a human donor. The functional annotation characterized general lymphocytes and specific B and T cell functions, indicating the precision of this workflow for clinical samples.

Conclusions: This robust and automated system should be broadly enabling for low-input proteomics for many researchers.

References: (1) Zhu, Y., et al. Nat. Commun. 2018, 9, 882

P235

In-depth single-cell proteome profiling of neuronal subtypes from human spinal tissue

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Topic: Single Cell Proteomics

Introduction: Single-cell analysis can reveal cellular heterogeneity that is obscured by bulk measurements. Recently developed nanoPOTS workflow coupled with ultrasensitive mass spectrometry has enabled single-cell proteomics analysis and discovered molecular mechanisms related to various disease and developmental processes (1-3).

Methods: Single motor neurons (MNs) and interneurons (INs) from fresh-frozen human spinal tissue were isolated by laser capture microdissection and processed with nanoPOTS workflow. The data were acquired on Thermo Orbitrap Eclipse Tribrid MS with the FAIMS Pro interface. Protein identification and quantitation were performed with Proteome Discoverer 2.5 and Perseus 1.6.7.0.

Results: Single motor neurons and interneurons microdissected from human spinal tissues were processed with nanoPOTS and analyzed on the ultrasensitive mass spectrometer with gas-phase FAIMS fractionation. The workflow resulted in more than 1000 protein groups to be identified from each neuronal subtype with an FDR of 1% and without MS1-level feature matching. The identified protein groups from the two cell subtypes had an overlap of 77% and were readily differentiated by principal component analysis. Among the 1118 quantifiable protein groups from the combined datasets, 39 were differentially enriched in MNs relative to INs, including proteins relevant to established MN functions. Gene ontology (GO) enrichment analyses on the subset of enriched-in-MN proteins revealed over-representation of proteins associated with RNA processing and alternative splicing, RNA metabolism, and post-transcriptional regulation of gene expression that is consistent with the established MN IHC markers and RNA expression data.

Conclusion: These data demonstrate the ability of unbiased proteomic profiling to differentiate neuronal subpopulations at the single-cell level and identify differentially expressed proteins and pathways.

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P236

Label-Free Proteomics Performance with New Orbitrap Exploris 480 mass spectrometer with Single-Cell Sensitivity

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Topic: Single Cell Proteomics

Introduction

LC-MS-based proteomics, a powerful technique for identification and quantification of peptides and proteins in complex samples. needs to provide robustness to analyze 1000s of samples without compromising on proteome coverage and quantitation performance. Here we demonstrate data reproducibility across different laboratories on the new Orbitrap Exploris 480 MS coupled to an High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) Interface. The sensitivity of the Orbitrap Exploris 480 MS was evaluated in a data dependent label free method from just a single Hela cell to 5000 HeLa cells with great quantitation accuracy across a wide range using a HeLa:Yeast spike-in experiment.

Methods

Single HeLa cells isolated via fluorescence-activated cell sorting, processed on Nanodroplet Processing in One Pot for Trace Samples (nanoPOTS) platform and Pierce HeLa digest in a range of 0.2-1000ng were analyzed to evaluate instrument sensitivity with different throughputs (30,60, 90 and 120 min gradients) on either the UltiMate™ 3000 RSLCnano (single cells) or EASY-nLC 1200 coupled to an Orbitrap Exploris 480 MS with FAIMS Pro interface. Label free, quantitation performance was evaluated to demonstrate instrument sensitivity and methods reproducibility.

Preliminary data

The performance of this new benchtop mass spectrometer was evaluated in a data-dependent acquisition (DDA) for sample injection amounts of just a single Hela cell to 5000 HeLa cells (~1ug). This instrument sensitivity enables identification of ~7000 protein groups with 5.5 order of magnitude dynamic range from only a 200 ng of bulk HeLa digest and great replicate reproducibility and ~800 protein groups identification from a single Hela cell in 2hr gradient. The method performance and reproducibility were also evaluated across different instruments located in different laboratories around the world with great reproducibility in peptide and protein identification.

Novel aspect

Robust and sensitive Orbitrap Exploris 480 MS, providing throughput and sensitivity needed for label-free proteomics analysis.

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