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Abstract Book

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Invited Speaker Presentation Summaries (PT, PL, KN,)

PT01.02
From Mass Spectrum to Protein

Prof. David Tabb
Institut Pasteur

These three discussions cover the basics of MS/MS identification: database search, FDR control, and protein inference. It is intended for researchers who are new to the field of proteomic identification.

PT01.03
Quantitative and Targeted Proteomics

Prof. Michael MacCoss
University of Washington

Dr. MacCoss has been working in quantitative mass spectrometry for >25 years. His presentation won't be specific to any type of quantitation but will be general to all types of quantitative proteomics.

PT03.02
Complex Centric Proteome Profiling

Dr. Isabell Bludau
Max Planck Institute of Biochemistry

Proteins are major effectors and regulators of biological processes and can elicit multiple functions depending on their interaction with other proteins. We have developed an integrated experimental and computational approach for detecting in parallel hundreds of protein complexes, as well as changes in their composition and abundance between samples, in a single operation. The method consists of size exclusion chromatography (SEC) to fractionate native protein complexes, SWATH/DIA mass spectrometry to precisely quantify the proteins in each SEC fraction, and the computational framework CCprofiler to detect and quantify protein complexes by complex-centric analysis. Extending this approach to the peptide-level further enables the investigation of proteoform-specific complex integration. Overall, the method provides information about protein complex assemblies within and across conditions at proteoform resolution and on an unprecedented, system wide scale.

PT05.01

Exploring the 'Social Network' within a Human Cell

Dr. Edward Huttlin
Harvard Medical School

The proteome may be viewed as a dynamic network of complexes, organelles, and signaling pathways that drive cellular function. Knowing the complete set of physical protein-protein interactions that underly these assemblies –the interactome– can thus reveal both systems-level proteome organization and physical and functional properties of individual proteins. For several years we have systematically profiled protein interactions in human cells using affinity-purification mass spectrometry (AP-MS). To date we have completed over 15,000 pull-downs, profiling interactions for most human proteins in two cell lines and revealing over 160,000 interactions to form two context-specific models of the human interactome, collectively called BioPlex. In this talk I will summarize our large-scale AP-MS platform and describe how we have used BioPlex to 1) explore structural organization of the human interactome; 2) derive functional insights and disease associations for poorly characterized proteins; and 3) explore context-specific interactome remodeling by comparing cell-line-specific interaction networks.

PT05.03

(Large-scale) Multi-omics Data Analysis

Dr. Johannes Griss
Medical Universtiy Of Vienna

ReactomeGSA is a pathway analysis platform to simplify multi-omcis, multi-species dataset comparisons.

PL02.02

Increasing the Sensitivity, Reliability, Reproducibility and Throughput of Single-Cell Proteomics

Assoc. Prof. Nikolai Slavov
Northeastern University

Current single-cell mass-spectrometry (MS) methods can quantify thousands of peptides per single cell while detecting peptide-like features that may support the quantification of 10-fold more peptides. This 10-fold gain might be attained by innovations in data acquisition and interpretation even while using existing instrumentation. This perspective discusses possible directions for such innovations with the aim to stimulate community efforts for increasing the coverage and quantitative accuracy of single proteomics while simultaneously decreasing missing data. Parallel improvements in instrumentation, sample preparation and peptide separation will afford additional gains. Together, these synergistic routes for

innovation project a rapid growth in the capabilities of MS based single-cell protein analysis. These gains will directly empower applications of single-cell proteomics to biomedical research.

PL03.01

3D Proteomics: From Structural to Functional Screens

Prof. Paola Picotti
ETH Zürich, Institute Of Molecular Systems Biology

Proteomics has been broadly applied to detect changes in protein levels in response to perturbations and derive information on altered pathways. Beyond protein expression changes, however, biological processes are also regulated by events such as intermolecular interactions, chemical modification and conformational changes. These events do not affect protein levels and therefore escape detection in classical proteomic screens. I will present how a global readout of protein structure can detect various types of protein functional alterations concomitantly. The approach, relying on the LiP-MS technique, monitors structural changes in thousands of proteins simultaneously in situ. It captures enzyme activity changes, allosteric regulation, phosphorylation and protein complex formation and pinpoints regulated functional sites, thus substantially expanding the coverage of proteomic analyses and supporting the generation of mechanistic hypotheses. Applications of this approach include the study of complex phenotypes, the identification of disease biomarkers and drug target deconvolution.

PL03.02

Structural Biology on the Proteome-Wide Scale Using Protein Footprinting

Dr. Lisa Jones
University Of Maryland

Lisa Jones is an Associate Professor from the University of Maryland. The title of her talk is Structural Biology on the Protoeme-Wide Scale using Protein Footprinting.

PL05.01

Thoughts on the Future of Proteomics

Prof. Michael Snyder
Stanford School Of Medicine

I will present the latest in using big data to improve healthcare and the results of the hPOP project, which is an HPP project to proifle people across the globe.

PL05.02

Exploring Uncharted Facets of the Proteome

Prof. Ruedi Aebersold
ETH Zurich

Mass spectrometry based proteomics has largely focused on confidently identifying and quantifying minimally one protein product from every coding gene. However, the proteome contains a wealth of additional information that is functionally highly relevant and remains essentially uncharted. Such information includes the resolution of products of specific genes to the level of proteoforms, the identification of PTM's and the organization of proteins into complexes and interaction networks. In this presentation we will discuss mass spectrometry-based methods to chart facets of the proteome beyond protein identification and quantification and discuss new approaches to advance from statistical associations towards causal relationships. It can be expected that these developments will further increase the significance of proteomics in basic and translational biology.

KN02.02

A Protein-Based Classifier for Thyroid Nodule Diagnosis

Dr. Tiannan Guo
Westlake University

Determination of malignancy in thyroid nodules remains a major diagnostic challenge. Here we report the feasibility and clinical utility of developing an AI-defined protein-based biomarker panel for diagnosing thyroid nodules: based initially on formalin-fixed paraffin-embedded (FFPE), and further refined for fine-needle aspiration (FNA) tissue specimens of minute amounts which pose technical challenges for other methods. We first developed a neural network model of 19 protein biomarkers based on the proteomes of 1724 FFPE thyroid tissue samples from a retrospective cohort of 578 patients. This classifier achieved over 91% accuracy in the discovery set for classifying malignant thyroid nodules. This classifier was externally validated by blinded analyses in a retrospective cohort of 288 nodules (89% accuracy; FFPE) and a prospective cohort of 294 FNA biopsies (85% accuracy) from six independent clinical centers. This study shows that integrating high-throughput proteomics and AI technology in multi-center retrospective and prospective clinical cohorts facilitates precise disease diagnosis which is otherwise difficult to achieve by other methods.

KN03.02

MONTE: A Multiomics Suite Enabling Serial Immunopeptidome, Ubiquitylome, Proteome, Phosphoproteome, Acetylome Analyses of Sample-Limited Tissues

Dr. Steven Carr
Broad Institute of MIT and Harvard

Multiomic characterization of patient tissues provides insights into the function of different biological pathways in the context of disease. Much work has been done to serialize proteome and post-translational modification (PTM) analyses to conserve precious patient samples. However, characterizing clinically relevant tissues with multi-ome workflows that have distinct sample processing requirements remains challenging. To overcome the obstacles of combining enrichment workflows that have unique input amounts and utilize both label free and chemical labeling strategies, we developed a highly-sensitive multi-omic networked tissue enrichment (MONTE) workflow for the full, deepscale analysis of HLA-I and HLA-II immunopeptidome, ubiquitylome, proteome, phosphoproteome and acetylome all from the same tissue sample.

KN05.02

Using Large Data Approaches to Diagnose and Drug Dementia

Assoc. Prof. Judith Steen
Boston Children's Hospital

Tau aggregation in the brain is a hallmark of Alzheimer's Disease and correlates with cognitive decline. While post-translational modifications (PTMs) have been associated with pathological Tau, a large-scale, in-depth quantitative characterization of Tau has not been performed. Quantitative mass spectrometry studies of Tau identified 90 conventional PTMs in post-mortem human brain tissue from over 200 human tauopathy patients, including Alzheimer's disease and age-matched controls. Correlation analyses with abundance of Tau identified key Tau domains and PTMs important to pathology. In addition, cross-referencing of clinical data showed that the Tau PTMs accumulate with disease progression in an ordered fashion. We further defined specific molecular features associated with Tau prion-like seeds in cell-based aggregation as well fibrilization assays. These detailed analyses of Tau allow us to define the chemical nature of Tau associated with its aggregation in human disease and the means to prevent the prion-like spread of aggregation.

KN06.02

Exploring Drug-Target Interaction Using Label-Free Darts and LC-MS/MS Method and Its Translational Impact

Prof. HoJeong Kwon
Yonsei University

Exploring protein targets of drugs and deciphering the specific mechanisms-of-action at the molecular level of these interactions are crucial steps in the development of drugs to treat human diseases. We have developed target protein identification methods including conventional affinity chromatography using labeled small molecules as well as recent methods using label-free small molecules such as Drug Affinity Responsive Target Stability (DARTS) and Cellular Thermal Shift Assay (CESTA) in combination with LC-MS/MS analysis to identify the direct binding proteins of drugs. The direct interaction between drug and the target protein is validated via bio-physical, and bio-informatics methods. Moreover, biological relevancy of this "drug-target" interaction is verified through genetic modulation that facilitates structure based better drug development. In this presentation, our studies on target identification of drug-target interaction for exploring new mechanism studies and translational impact will be presented by introducing our case studies of protein target identification and validation of natural products and clinical drugs perturbing autophagy.

Early Career Researcher (ECR) Abstracts

ECR.01

Global and Site-specific Effect of Phosphorylation on Protein Turnover

Dr. Yansheng Liu[1,2]
[1]Yale University School of Medicine, [2]Yale Cancer Biology Institute

To date, the effects of specific modification types and sites on protein lifetime have not been illustrated at large scale. Here, we describe a proteomic method, DeltaSILAC, to quantitatively assess the impact of site-specific phosphorylation on the turnover of thousands of proteins in live cells. Based on the accurate and reproducible mass spectrometry, a pulse labeling approach using stable isotope-labeled amino acids in cells (pSILAC), phosphoproteomics, and a novel peptide-level matching strategy, our DeltaSILAC profiling revealed a global, unexpected delaying effect of many phosphosites on protein turnover. We further found that phosphorylated sites accelerating protein turnover are functionally selected for cell fitness, enriched in Cyclin-dependent kinase substrates, and evolutionarily conserved, whereas the Glutamic acids surrounding phosphosites significantly delay protein turnover. Our method represents a generalizable approach and provides a rich resource for prioritizing the effects of phosphorylation sites on protein lifetime in the context of cell signaling and disease biology.

ECR.02

The Global Phosphorylation Landscape of SARS-CoV-2 Infection

Dr. Mehdi Bouhaddou[1], Danish Memon[2], Bjoern Meyer[3], Kris M. White[4], Veronica V. Rezelj[3], Miguel Correa Marrero[2], Benjamin J. Polacco[1], James E. Melnyk[1], Svenja Ulferts[5], Robyn M. Kaake[1], Jyoti Batra[1], Lorena Zuliani-Alvarez[1], Lucy G. Thorne[6], Ann-Kathrin Reuschl[6], Greg J. Towers[6], Clare Jolly[6], Elizabeth R. Fischer[7], Robert Grosse[5], Adolfo Garcia-Sastre[4], Marco Vignuzzi[3], Jeffrey R. Johnson[1], Kevan M. Shokat[1], Danielle L. Swaney[1], Pedro Beltrao[2], Nevan J. Krogan[1]
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Cellular signaling, as regulated by phosphorylation, plays a role in most, if not all, aspects of biological life. Mass spectrometry-based proteomics technology permits the simultaneous quantification of tens of thousands of phosphorylation sites from a single sample, acting as a code for the biochemical signaling pathways and cellular processes regulated in response to a stimulus. A major challenge in omics data science lies in extracting biological insight from complex datasets, for which computational data integration approaches are well poised. Subsequently, how best to map biological insight to novel therapeutic approaches?

In a recent study performed in collaboration with the Quantitative Biosciences Institute Research Group (QCRG), we performed quantitative mass spectrometry-based phosphoproteomics of SARS-CoV-2 infection in Vero E6 cells, which revealed dramatic rewiring of phosphorylation on host and viral proteins (Bouhaddou et al., Cell 2020). By pairing network bioinformatics with experimental validation, we revealed SARS-CoV-2 infection promoted casein kinase II (CK2) and p38 MAPK activation, production of diverse cytokines, and shutdown of mitotic kinases, resulting in cell cycle arrest. Infection also stimulated a marked induction of CK2-containing filopodial protrusions possessing budding viral particles. Eighty-seven drugs and compounds were identified by mapping global phosphorylation profiles to dysregulated kinases and pathways, revealing inhibition of the p38, CK2, CDK, AXL, and PIKFYVE kinases to possess antiviral efficacy, representing potential COVID-19 therapies.

Building on the methods and insights gained, we extended our proteomics and computational pipeline to studying emerging SARS-CoV-2 variants of concern, which represent prominent global health hazards (Thorne, Bouhaddou, Reuschl, Zuliani-Alvarez et al., bioRxiv 2021). By integrating global proteomics and RNA sequencing, we revealed the Alpha variant (B.1.1.7) to strongly antagonize innate immune signaling, which could explain an increased transmission rate by lowering the threshold needed to establish infection.

ECR.03

Benefits of Chemical Sugar Modifications Introduced by Click Chemistry for Glycoproteomic Analyses

Beatriz Calle[2,3], Ganka Bineva-Todd[2,3], Andrea Marchesi[2,3], Helen Flynn[2], Mattia Ghirardello[4], Omur Tastan[2], Chloe Roustan[2], Junwon Choi[5], M. Carmen Galan[4], Benjamin Schumann[2,3], Dr. Stacy Malaker[1]
[1]Yale University, [2]The Francis Crick Institute, [3]Imperial College London, [4]University of Bristol, [5]Ajou University

Mucin-type O-glycosylation is among the most complex post-translational modifications. Despite mediating many physiological processes, O-glycosylation remains understudied compared to other modifications, simply because the right analytical tools are lacking. In particular, analysis of intact O-glycopeptides by mass spectrometry is challenging for several reasons; O-glycosylation lacks a consensus motif, glycopeptides have low charge density which impairs ETD fragmentation, and the glycan structures modifying the peptides are unpredictable. Recently, we introduced chemically modified monosaccharide analogues that allowed selective tracking and characterization of mucin-type O-glycans after bioorthogonal derivatization with biotin-based enrichment handles. In doing so, we realized that the chemical modifications used in these studies have additional benefits that allow for improved analysis by tandem mass spectrometry. In this work, we built on this discovery by generating a series of new GalNAc analogue glycopeptides. We characterized the mass spectrometric signatures of these modified glycopeptides and their signature residues left by bioorthogonal reporter reagents. Our data indicate that chemical methods for glycopeptide profiling offer opportunities to optimize attributes such as increased charge state, higher charge density, and predictable fragmentation behavior.

Keynote Session Abstracts (KN)

KN01.03

Profiling SARS-CoV-2 HLA-I Peptidome Reveals T Cell Epitopes From Out-of-Frame ORFs

Dr. Susan Klaeger[1], Shira Weingarten-Gabbay[1,2], Siranush Sarkizova[1], Leah R. Pearlman[1], Da-Yuan Chen[3,4], Kathleen M.E. Gallagher[5,6], Matthew R. Bauer[1,7], Hannah B. Taylor[1], W. Augustine Dunn[8], Christina Tarr[8], John Sidney[9], Suzanna Rachimi[1], Hasahn L. Conway[3,4], Katelin Katsis[5], Yuntong Wang[8], Del Leistritz-Edwards[8], Melissa R. Durkin[8], Christopher H. Tomkins-Tinch[1,2], Yaara Finkel[10], Aharon Nachshon[10], Matteo Gentili[1], Keith D. Rivera[1], Isabel P. Carulli[11], Vipheaviny A. Chea[11], Abishek Chandrashekar[12], Cansu Cimen Bozkus[13], Mary Carrington[14,15], MGH COVID-19 Collection & Processing Team[16], Nina Bhardwaj[13], Dan H. Barouch[6,12,14,17], Alessandro Sette[9,18], Marcela V. Maus[5,6,19,20], Charles M. Rice[21], Karl R Clauser[1], Derin B. Keskin[1,11,22,23], Daniel C. Pregibon[8], Nir Hacohen[1,20,24], Steven A. Carr[1], Jennifer G. Abelin[1], Mohsan Saeed[3,4], Pardis C. Sabeti[1,2,17,25,26]
[1]Broad Institute of MIT and Harvard, [2]Department of Organismic and Evolutionary Biology, Harvard University, [3]Department of Biochemistry, Boston University School of Medicine, [4]National Emerging Infectious Diseases Laboratories, Boston University, [5]Cellular Immunotherapy Program and Cancer Center, Massachusetts General Hospital, [6]Harvard Medical School, [7]Harvard Program in Biological and Biomedical Sciences, Harvard Medical School, [8]Repertoire Immune Medicines, [9]Center for Infectious Disease and Vaccine Research, La Jolla Institute for Immunology (LJI), [10]Department of Molecular Genetics, Weizmann Institute of Science, [11]Translational Immunogenomics Laboratory, Dana-Farber Cancer Institute, [12]Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, [13]Department of Hematology and Medical Oncology, Icahn School of Medicine at Mount Sinai Hospital, [14]Ragon Institute of MGH, MIT and Harvard, [15]Basic Science Program, Frederick National Laboratory for Cancer Research in the Laboratory of Integrative Cancer Immunology, National Cancer Institute, [16]Massachusetts General Hospital, Harvard Medical School, [17]Massachusetts Consortium on Pathogen Readiness, [18]Department of Medicine, Division of Infectious Diseases and Global Public Health, University of California San Diego, [19]Klarman Cell Observatory, Broad Institute of MIT and Harvard, [20]Department of Medicine, Massachusetts General Hospital, [21]Laboratory of Virology and Infectious Disease, The Rockefeller University, [22]Health Informatics Lab, Metropolitan College, Boston University, [23]Department of Medical Oncology, Dana-Farber Cancer Institute, [24]Center for Cancer Research, Massachusetts General Hospital, [25]Department of Immunology and Infectious Disease, Harvard T.H. Chan School of Public Health, [26]Howard Hughes Medical Institute

Introduction:
T cell-mediated immunity plays an important role in controlling SARS-CoV-2 infection; yet the repertoire of naturally processed and presented viral epitopes on HLA class I remains uncharacterized. Viruses manipulate the antigen presentation machinery and the dynamics of viral protein expression and antigen presentation vary during the course of infection. These effects are currently not captured by HLA-I binding predictors. Mass spectrometry (MS) based HLA-I immunopeptidomics provides direct measurements of naturally presented peptides upon infection and can deepen our understanding of T cells responses to SARS-CoV-2.
Methods:
A549 and HEK293T cells were harvested at 0, 3, 6, 12, 18 and 24 hours after SARS-CoV-2 infection. HLA-I complexes were immunoprecipitated with W6/32 antibody, peptides were acid eluted and analyzed by

high-resolution LC-MS/MS (Orbitrap Exploris with FAIMS). Deep proteome measurements were performed from the same samples. Mass spectra were interpreted with the Spectrum Mill software package and a human protein database including annotated proteins and 23 unannotated open reading frames in the SARS-CoV-2 genome.

Results:

We identified viral HLA-I peptides derived from canonical and internal out-of-frame ORFs in Spike and Nucleocapsid. Whole proteome measurements suggested that time of viral protein expression correlated with HLA-I presentation and immunogenicity and that SARS-CoV-2 interferes with the proteasomal pathway. Peptides derived from out-of-frame ORFs elicit T cell responses in immunized mice and COVID-19 patients as evaluated by ELISpot and multiplexed barcoded tetramer assays. Computational predictions and biochemical binding assays demonstrate that detected HLA-I peptides can be presented by additional HLA-I alleles beyond the nine alleles tested in our study.

Conclusions:

In conclusion, we report the first HLA-I immunopeptidome of SARS-CoV-2 in two cell lines at different times post-infection using mass spectrometry. The discovery of out-of-frame ORF epitopes and other biological insights will facilitate selection of peptides for immune monitoring and aid in design of efficacious vaccines.

KN01.04

SARS-CoV-2 Structural Coverage Map Reveals Viral Protein Assembly, Mimicry, and Hijacking Mechanisms

Prof. Sean ODonoghue[1,2,3], Dr Andrea Schafferhans[4,5], Ms Neblina Sikta[1], Mr Christian Stolte[1], Ms Sandeep Kaur[1,3], Dr Bosco Ho[1], Mr Stuart Anderson[2], Dr James Procter[6], Dr Christian Dallago[5], Dr Nicola Bordin[7], Dr Matt Adcock[2], Prof Burkhard Rost[5]
[1]Garvan Institute Of Medical Research, [2]CSIRO Data61, [3]School of Biotechnology and Biomolecular Sciences, UNSW, [4]Weihenstephan-Tr. University of Applied Sciences, [5]Technical University of Munich, [6]The University of Dundee, [7]University College London

Introduction
The past 18 months have seen sustained efforts to elucidate the 3D structure of all SARS-CoV-2 proteins, both by experimental and modelling techniques. However, most modelling studies have focused on deriving only a few structural states for each viral protein; thus, to date, there has been no published, systematic analysis examining all structural states with supporting structural evidence.

Methods
To address this, we used sequence profiles (HHblits) to systematically model all SARS-CoV-2 protein sequences, testing against all protein structures in the PDB. To make the resulting 3D models more accessible, we also devised a structural coverage map, a novel, one-stop visualization concept that provides an insightful overview of what is — and is not — known about the 3D structure of the viral proteome. The coverage map and models were integrated into Aquaria (O'Donoghue et al., Nature Methods, 2015).

Results
We found 2,060 structural models, of which 880 were determined for organisms other than SARS-CoV-2; collectively, these models span 69% of the viral proteome. These models provided insight into the assembly of the replication and translation complex, and suggest that 6% of the viral proteome mimics human proteins. In addition, the models suggest that 7% of the viral proteome hijacks human proteins, resulting in reversal of post-translational modifications (ADP ribosylation, ubiquitination, and ISGylation), blocking of host translation, and disabling of host defences. These results are summarized in the structural coverage map, which researchers can use online to find and explore 3D models corresponding to any of the 79 distinct structural states identified in this work.

Conclusions
In the ongoing COVID-19 pandemic, our Aquaria-COVID resource (https://aquaria.ws/covid) helps scientists use emerging structural data to understand the molecular mechanisms underlying coronavirus infection, and draws attention to the 31% of the viral proteome that remains structurally unknown or dark.

KN02.03

Beyond Fibril-Forming Proteins: Developing a More Holistic Approach on Amyloid Formation

Ms. Juliane Gottwald[1], Ms. Annelie Lux[1], Dr. Christian Treitz[2], Ms. Eva Linda Gericke[1], Dr. Hans-Michael Behrens[1], Prof. Andreas Tholey[2], Prof. Christoph Röcken[1]
[1]Department of Pathology, Christian-Albrechts-University Kiel, [2]Systematic Proteome Research & Bioanalytics, Institute of Experimental Medicine, Christian-Albrechts-University Kiel

Introduction: Amyloidoses are a group of diseases caused by protein misfolding and subsequent deposition of insoluble fibrils in diverse tissue sites. Until now, 36 fibril-forming proteins are known to cause amyloidoses with diverse clinical patterns and outcomes. Introduction of bottom-up proteomics enabled specific subtyping of amyloidosis for diagnostics but also provided insights into amyloid composition. Here, we present our proteomic approaches to work up this data and how we focus on amyloid constituents beyond the fibril-forming protein.

Methods: We investigated known amyloid-associated peptides by MALDI ion mobility separation mass spectrometry imaging (MALDI-IMS MSI) in human tissue samples. LC-MS/MS data of amyloid deposits from various tissue- and amyloidosis-types were collected and evaluated using R (1). Candidates of interest were further analysed by immunohistochemistry (IHC; 2) and LC-MS/MS.

Results: We visualised the distribution of known amyloid-associated peptides within various tissues by MALDI-IMS MSI. Aiming to discover new disease-specific contributors our systematic meta-analysis (1) showed that proteins in amyloid deposits can be classified into four amyloid protein categories (APC): fibrillary proteins found in the patient (APC1); potentially fibril-forming proteins found in other types of amyloidoses (APC2); disease-specific, non-fibril forming or amyloid signature proteins (APC3); normal tissue constituents and tissue remodeling indicators (APC4). Complement components including C9 were among our APC3 findings. Using IHC we demonstrated C9 to be as common in diverse tissue- and amyloidosis-types as the amyloid signature-protein apolipoprotein E (2). Additionally, LC-MS/MS identified a plethora of complement-associated proteins, which correlated with fibril-forming transthyretin in carpal tunnel.

Conclusions: Our categorisation provides a better overview of potentially important players in disease manifestation, progression, and clearance. Based on this, we unraveled an innate immune defense mechanism in amyloid deposits—the complement system.

(1) Gottwald, J. and Röcken, C. 2021 Crit Rev Biochem Mol Biol; 1-17
(2) Lux, A., et al. 2021 Amyloid; 1-10

KN02.04

Multiomics Identification and Validation of Novel Blood-Based Alzheimer's Disease Autoantibody Biomarkers.

Miren Alonso-Navarro[1,4], Ana Montero-Calle[1], Pablo San Segundo-Acosta[1], August Jernbom-Falk[2], María Garranzo-Asensio[1], Guillermo Solís-Fernández[1,3], Ana Guzmán Aránguez[4], Anna Månberg[2], Rodrigo Barderas[1]
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-Introduction
Alzheimer's disease (AD) is a progressive and chronic neurodegenerative disorder that affects wide areas of the cerebral cortex and hippocampus [1, 2]. It is the most common cause of dementia worldwide with a high socioeconomic impact. Since the definitive diagnosis of AD requires post-mortem verification, new approaches are necessary to identify diagnostic biomarkers and therapeutic targets of the disease [3]. Here, we aimed to identify AD-specific autoantibodies and autoantigens as blood-based biomarkers using a multiomics approach [4].
-Methods
Protein-epitope signature tag (PrESTs) protein microarrays and mass spectrometry-based methods were used for the identification of AD autoantibodies and their target proteins. Validation was performed by PrESTs beads-based, ELISA, Luminescence assays, WB and immunohistochemistry using serum and brain tissue samples from AD patients and controls and PrESTs or full-length recombinant proteins.
-Results
High-density (42,100) and low-density (384) PrEST planar arrays were used together with an immunoprecipitation protocol coupled to mass spectrometry (LC-MS/MS) analysis, using either frozen brain tissue or serum samples from AD patients and healthy individuals, for serum AD-related autoantibody and autoantigen identification.
After validation of the identified PrESTs target of autoantibodies, a candidate PrEST possessed a statistically significant higher seroreactivity in AD patients than in controls. Besides, two other AD-related seroreactive autoantigens were identified by immunoprecipitation followed by LC-MS/MS analysis.
Seroreactivity to both autoantigens and the candidate PrEST was further validated by ELISA and Luminescence assays using full-length recombinant proteins, showing the three autoantigens in combination a promising AD diagnostic ability. Besides, the three targets of autoantibodies showed altered protein levels in the brain of AD patients in comparison to controls as observed by WB or immunohistochemistry.
-Conclusions
Our results suggest that the multiomics approach is suitable for the identification of AD-related autoantigens and protein alterations related to the disease that could be useful as blood-based AD biomarkers.

KN03.03

In-depth Plasma Proteomics Profiling with ProteographTM Product Suite: A Performance Evaluation of Label Free and TMT Multiplexing Approaches

Dr. Alex Rosa Campos[1], Dr. Ramon Diaz Pena[1], Dr. Khatereh Motamedchaboki[2], Dr Aaron S Gajadhar[2], Dr Qiu Zuo Yang[2], Dr Lucy Williamson[2], Dr Tianyu Wang[2]
[1]*Sanford-Burnham-Prebys Medical Discovery Institute,* [2]*Seer, Inc.*

Human blood plasma is a widely accessible samples for assessing individual health status. However, the large dynamic range of circulating proteins combined with the vast proteoform diversities have precluded the comprehensive characterization of the plasma proteome in a high throughput manner. To address such challenges current plasma proteomics workflows combine immunodepletion of high abundance proteins, sample multiplexing approaches such as tandem mass tags (TMT) ,for relative and absolute quantitation, and peptide fractionation. Here we evaluate the performance of label- free versus TMT multiplexing LCMS data acquisition method with a set of control plasma samples processed with nanoparticle-based ProteographAssay Kit.

Pooled control human plasma were processed with Proteograph SP100 automated sample preparation instrument (Seer Inc.). Tryptic peptides were either directly analyzed by LC-MS/MS or labeled with one of the TMTpro reagents followed by peptide fractionation by high pH RP and LC-MS/MS analysis, comprised of a Proxeon EASY nanoLC system,C18 Aurora column (IonOpticks) coupled to an Orbitrap Fusion Lumos MS equipped with FAIMS Pro Interface (Thermo Fisher Scientific). All mass spectra from were analyzed with SpectroMine software (Biognosys).

Using an automated workflow, up to 16 biofluid samples can be processed and analyzed with LC-MS/MS with 24- or 48-hours workflows. TMT combine with peptide fractionation, resulted in 3,000 protein groups (~80% with 2 or more peptides) with 8 samples per day workflowand~ 1,800 proteins with a throughput of 16 samples per day (~75% with 2 or more peptides).
Approximately 86% of the features are detected across all 4 batches, Among the proteins detected in this dataset, numbers of low abundance cytokine signaling proteins were detected such as CD4, CD40L, CXCL2, members of TNF superfamily such as TNFSF13, TNFRSF6B and numerous MHC proteins. Many proteins detected are potential biomarkers for several diseases including 456 cancer-related proteins, in addition to 168 FDA-approved drug targets.

KN03.04

Next-generation Serology by Mass Spectrometry: Ig-MS Readout of the SARS-CoV-2 Antibody Repertoire

Dr. Rafael Melani[1], Dr. Benjamin Des Soye[1], Dr Jared Kafader[1], Dr Eleonora Forte[1], Dr Michael Hollas[1], Dr Voislav Blagojevic[1], Dr Fernanda Negrão[1], John McGee[1], Dr Bryon Drown[1], Cameron Lloyd-Jones, Dr Henrique Seckler[1], Dr Jeannie Camarillo[1], Dr Philip Compton[1], Dr Richard LeDuc[1], Bryan Early[1], Ryan Fellers[1], Dr Byoung-Kyu Cho[1], Dr Basil Baby Mattamana[1], Dr Young Goo[1], Dr Paul Thomas[1], Michelle Ash[2], Pavan Bhimalli[2], Lena Al-Harthi[2], Beverly Sha[2], Dr Jeffrey Schneider[2], Dr Neil Kelleher[1]
[1]Northwestern University, [2]Rush University Medical Center

Introduction: Traditional antibody detection methods inform the total amount of immunoglobulins generated in response to a given antigen, and they are unable to capture clonality. Individual ion mass spectrometry (I2MS) can provide mass distributions for extremely heterogeneous samples using low amounts of proteins. We used I2MS to develop Ig-MS and access at molecular resolution variable regions in antibodies light (LC) and heavy chains (HC).
Methods: Antibodies against the RBD-domain of SARS-CoV-2 spike protein were pulled down from the plasma of convalescent COVID-19 patients, vaccinated subjects, and uninfected individuals. Reduced antibody chains were analyzed by I2MS. The mass spectra were used to calculate Ion Titers (IT) and Degree of Clonality (DoC).
Results: Following the assay development and optimization, we applied Ig-MS to a small cohort of subjects, including seven COVID-19 hospitalized patients, three outpatients, and three uninfected individuals. We found that 1) IT from the two workflows was self-consistent and correlated with traditional colorimetric/fluorimetric and neutralization assays; 2) similar to other reports, there are significant differences in hospitalized patients versus outpatients/uninfected individuals in terms of titer; and 3) the DoC metric and LC mass patterns did not correlate with any conventional titer assay showing its uniqueness. Furthermore, we analyzed the antibody response in four immunized individuals at two-time points: after the first and the second shots. We found a significant increase both in antibody IT and DoC between the first and the second dose. Interestingly, fully vaccinated and COVID-19 hospitalized subjects showed comparable IT and DoC while outpatients had similar metrics to uninfected individuals.
Conclusions: For the first time, Ig-MS can molecularly resolve and visualize the surge of antibody proteoform diversity. The two new metrics can help to predict immune protection and disease severity. Furthermore, Ig-MSt is adaptable to any antigen to gauge immune responses to vaccination, pathogens, or autoimmune disorders.

KN04.03

Approaching a Non-invasive Diagnosis of Endometrial Cancer by the Analysis of Protein Biomarkers in Pap-Smears

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Introduction: Diagnosis of endometrial cancer (EC) is performed on approximately 14M women in US and Europe every year who present with abnormal vaginal bleeding. However, only 5-10% will have EC. The diagnostic process always requires minimally invasive or invasive samplings of the endometrium for pathological examination to diagnose/rule out EC. Here, we aim to decipher protein biomarkers in the fluid of cervical cytologies, i.e. pap-smears, to achieve a non-invasive diagnosis of EC.
Methods: The discovery phase consisted of a data-dependent acquisition (DDA) of cervical fluids from 60 patients (20 EC, 20 non-EC, 20 non-EC with cervical pathology), followed by a targeted verification by LC-PRM in cervical fluids of 234 patients (128 EC; 113 non-EC). A logistic regression model assessed by 10-k-fold cross validation was used to assess the power of protein panels to diagnose EC and differentiate between EC histological subtypes and grades. Furthermore, the highest performing biomarker was validated in an ELISA assay using the verification cohort. Analysis was performed using MaxQuant, Skyline, SPSS and R software.
Results: The discovery study determined 2,888 proteins contained in cervical fluids. Statistical analysis identified 75 significant proteins between EC and non-EC, and 58 were verified. Among those, 16 had an AUC > 0.75. A 3-protein panel allowed EC diagnosis with an AUC of 0.957 (93% sensitivity and 90% specificity). Additionally, a 5-protein panel was able to distinguish histological subtypes and grades with AUC values of 0.88 and 0.97, respectively. The best performing diagnostic protein was transferred to ELISA reaching an AUC of 0.927.
Conclusions: Proteomics applied to cervical fluids permitted to identify protein panels that permit a highly accurate non-invasive diagnosis of EC, in addition to the determination of histological subtypes and grades. Our results aim to impact the standard of care of EC diagnosis.

KN04.04

Health Surveillance Panel Multiplexed MRM-Based Protein Assay for the Identification of Multiple Biomarkers of Disease Severity in Human Coronary Disease

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Introduction
Health surveillance panel (HSP) is a targeted protein mass spectrometry-based multiplex protein assay. HSP assess nine physiological protein signatures, including inflammation and vascular dysfunction, based on the precise quantitation of 60 plasma proteins. Analysis of plasma from the initial 605 individuals recruited to the Multi-Ethnic Study of Atherosclerosis (MESA)1, a cross sectional research study composed of 6814 asymptomatic men and women, focused on defining subclinical cardiovascular disease. Assay performance and comparison to several known plasma biomarkers of coronary health (e.g. CRP, VWF) were assessed. The goal is to determine mechanistic protein signature reflecting risk factors predictive of the progression to subclinical and clinically overt cardiovascular disease based on their subsequent long-term clinical outcomes.

Methods
Fifty micrograms of each plasma sample were reduced, alkylated, then trypsin digested for 16 hours with heavy labeled CRP protein as the digestion control. Stable Isotope labeled peptides were used as protein surrogates. A total of 114 tryptic peptides were separated on a 22.3 min gradient using U3000 (Thermo) at high flow and their abundance was measured using 6500+ Triple Quadrupole (Sciex) with 30 minutes acquisition time. Data was processed using SciexOS v2.1. Statistical analysis was performed using in-house scripts.

Results.
Peptide stability (over 96 hours), linearity, inter and intra-day variables were established for each peptide in different plasma matrices. On average, peak widths were < 15 sec, matrix dependent recovery for each peptide was > 80%, matrix effect <30% and the linearity $r^2 > 0.9$. The key performance measure remained high during the 605 samples acquisition. To increase throughput, two Triple Quadrupoles were harmonized with a $r^2 > 0.99$.

Conclusion. Optimization of the HSP was carried out prior to running initial 605 plasma samples from the MESA cohort. Assay precision and correlation to known immunoassay data on several analytes were determined.

References
(1) https://www.mesa-nhlbi.org/aboutMESA.aspx

KN05.03

Parallel Proteomic and Microscopy Analyses Identify Protein Networks Associated with Synapse Loss Across Aging in the Human Brain

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Introduction
Aging in humans is associated with cognitive decline and increased risk of neurodegenerative disorders such as Alzheimer’s Disease. Synapse loss is believed to contribute to both. Synapses are maintained by complex protein networks, alterations to which likely contribute to synapse loss in aging.
Methods
Here, we utilized multiple label confocal microcopy to measure synapse number and size in concert with TMT-based quantitative proteomics to survey protein expression and synaptosome protein levels in cortical (precuneus) grey matter from 98 of subjects free of neurological or psychiatric diagnoses, with ages evenly distributed between 23-96, balanced for sex and postmortem interval.
Results
Homogenate and synaptosome proteomes were robustly altered during aging. Linear regression found that 1,634/5,033 quantified homogenate proteins and 917/4,754 quantified synaptosome proteins were significantly associated with age ($q < 0.05$). Of the 1,634 homogenate proteins associated with aging, 584 have been found to be altered in Alzheimer’s Disease.
Age was negatively correlated with large synapses ($r = -0.36$, $p = 0.00025$), while no association with small or medium synapses was observed. Large synapses are considered more mature and functional, implying that aging results in the loss of long-term cortical circuits.
WGCNA identified 18 homogenate protein modules and 17 synaptosome protein modules. Of these, two homogenate and one synaptosome module statistically mediated the effects of age on the large synapses. The spine-mediating homogenate modules were enriched for trans-synaptic signaling and synaptic organization terms.
Mediation analysis of individual proteins identified many previously implicated in neuropsychiatric developmental disorders (e.g. KALRN) as well as novel proteins not previously implicated in synaptic biology (e.g. MIOS).
Conclusions
This work provides a rich dataset of proteome alterations that statistically mediate spine loss in aging and provides a basis to begin nominating novel proteins and pathways for targeted intervention in synapse loss in normal aging and Alzheimer’s Disease.

KN05.04

Proteomic/PTMomic Screening of Human Parkinson's Disease IPSC-derived Neurons Identifies Disease Phenotypes and Potential Therapeutic Targets

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Introduction: The development of effective therapeutics for Parkinson's disease (PD) is hampered by our limited mechanistic understanding of the initiation and progression of the disease. However, recent research suggests that axonal degeneration could be a key event at early stages. We set out to identify common pathogenic mechanisms arising from PD-causing mutations in PARK2 and GBA, two genes with very distinct roles in the neurons. PARK2 encodes the E3 ligase parkin, which is vital for mitophagy, whereas GBA encodes glucocerebrosidase, an important lysosomal enzyme. By studying the effect of these mutations in human induced pluripotent stem cell (iPSC)-derived neurons, we aimed to elucidate the early molecular perturbations underlying the disease.
Methods: Utilising a large-scale proteomic and post-translational modification (PTM) screening approach, we compared PD patient iPSC-derived neurons to healthy controls and identified protein level changes and alterations in PTMs including phosphorylation, reversible cysteine modification and sialylation, which are all key in regulating protein function and -localisation in neurons.
Results: Network analysis of the proteomic and PTMomic changes revealed perturbations in cell survival, migration and neurite outgrowth in neurons with PARK2 knockout (1,2). The small GTPase RhoA was identified as a key upstream regulator, and by inhibiting RhoA signalling the migration and neurite outgrowth phenotypes could be rescued (2). Neurite outgrowth defects were similarly confirmed in GBA patient-derived neurons. Applying a glucocerebrosidase chaperone significantly improved neurite outgrowth in the patient-derived neurons (3).
Conclusion: Our study points to neuritic defects as an early, common disease mechanism in PD and demonstrates how proteomic/PTMomic screening of patient iPSC-derived neurons can reveal disease phenotypes and highlight targets with potential for therapeutic intervention.

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KN06.03

Proteomic Markers Predict Response to Methotrexate in Patients with Early Rheumatoid Arthritis

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Introduction: Methotrexate (MTX) is the first-line therapy for patients with rheumatoid arthritis (RA), despite of the risk of treatment failure and side-effects. We aimed to identify and validate circulating biomarkers useful as a precision medicine tool to predict response to MTX in early RA patients.

Methods: Disease activity was determined in patients belonging to the Pathobiology of Early Arthritis Cohort (PEAC) before and after six months of treatment with MTX and classified into responders and non-responders following the EULAR criteria. Serum samples at baseline (N=60) were analyzed by nLC-MS/MS using a SWATH strategy on a TripleTOF MS. Bioinformatic analyses were performed on the quantitative data in a training set of 30 samples. Then, the outcomes were validated by a two-stage support vector machine (TSSVM) in an independent data set of 24 samples. A custom antibody microarray was developed and used, together with ELISA, to replicate the results.

Results: 229 proteins were identified and quantified in the serum samples by MS/MS in both screening and validation sets. Data were pre-processed by PCA for dimension reduction and analyzed by machine learning tools, leading to the definition of a panel of 8 proteins that discriminates at baseline the groups of responders and non-responders to MTX after 6 months, with very high accuracy (sensitivity/specificity = 0.88/0.94). Following an analogous workflow, data generated by immunoassays techniques showed an area under the curve (AUC) of 0.78.

Conclusions: A panel of 8 circulating proteins useful to predict the response to MTX therapy in early RA patients has been identified and validated by proteomics. Further replication in independent cohorts is needed to establish the clinical utility of this tool for precision medicine strategies.

KN06.04

Warp-speed Selectivity Profiling of Small Molecule Inhibitors Using µSPE Chip-based CE-MS with PRM-LIVE Acquisition on An Ion Mobility Mass Spectrometer

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INTRODUCTION
Deubiquitinases (DUBs) comprise ~100 enzymes which cleave ubiquitin from substrates to regulate key aspects of human physiology. Pharmacologic inhibition of DUBs can have therapeutic benefits in autoimmune disorders, oncology, neurodegeneration, and other indications. Similar to the kinase field ~25 years ago, there are currently no approved DUB-targeting drugs and most preclinical small molecules are low-potency and/or multi-targeted. To facilitate high-throughput identification of new small molecule inhibitors that target the subset of ~85 cysteine protease DUBs, we developed a novel CE microchip (ZipChip) containing an on-board C18 bed for sample preconcentration. We coupled this ballistic separation platform to our new PRM-LIVE acquisition on timsTOF Pro to enable warp-speed activity-based selectivity profiling (ABPP) of novel small molecule inhibitors against endogenous DUBs.

METHODS
Microfluidic chips were constructed with a reservoir of C18 resin (µSPE) for on-chip peptide preconcentration. Competitive binding assays between DUB inhibitors and DUB-reactive activity probe were performed in human cell extracts, followed by enrichment, digest, TMT labeling and PRM-LIVE analysis on µSPE-ZipChip-timsTOF Pro.

RESULTS
We performed PASEF-DDA analyses on the µSPE-ZipChip timsTOF Pro to identify 279 peptides that mapped uniquely to 43 DUBs, or 57% of the cysteine protease DUBs predicted based on gene expression. We co-incubated small molecule DUB inhibitors with the DUB activity-based probe in a competitive binding assay. After pulldown enrichment, we spiked-in protein standards at known ratios, digested with trypsin, and labeled peptides from each inhibitor-treated condition with TMT 6-plex reagents. PRM-LIVE on the µSPE-ZipChip timsTOF Pro platform reproducibly detected all 279 peptides spanning 43 endogenous DUBs. Our PRM-LIVE data recapitulated binding profiles of these inhibitors in ballistic 10-min acquisition, with TMT quantification accuracy ± 10% based on spike-in protein standards.

CONCLUSIONS
µSPE-CE plus highly multiplexed PRM-LIVE provides warp-speed and accurate selectivity profiling of new inhibitors against DUBs as an emergent class of drug targets.

KN07.03

Ionbot: A Novel, Innovative and Sensitive Machine Learning Appr--oach to LC-MS/MS Peptide Identification

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Mass spectrometry-based proteomics generates vast amounts of signal data that require computational interpretation to obtain peptide identifications. Dozens of algorithms for this task exist, but all exploit only part of the acquired data to judge a peptide-to-spectrum match (PSM), ignoring important information such as the observed retention time and fragment ion peak intensity pattern. Moreover, only few identification algorithms allow open modification searches that can substantially increase peptide identifications.

We here therefore introduce ionbot, a novel open modification search engine that is the first to fully merge machine learning with peptide identification. This core innovation brings the ability to include a much larger range of experimental data into PSM scoring, and even to adapt this scoring to the specifics of the data itself. As a result, ionbot substantially increases PSM confidence for open searches, and even enables a further increase in peptide identification rate of up to 30% by also considering highly plausible, lower-ranked, co-eluting matches for a fragmentation spectrum. Moreover, the exclusive use of machine learning for scoring also means that any future improvements to predictive models for peptide behavior will also result in more sensitive and accurate peptide identification.

KN07.04

Systematic Detection of Functional Proteoform Groups from Bottom-up Proteomic Datasets

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Introduction: Cells can create multiple distinct but related proteins per coding gene – so-called proteoforms – that expand their functional capacity. The investigation of proteoforms is a major challenge in bottom-up proteomics, due to peptides being measured rather than intact proteoforms. Here we present COPF, a tool for COrrelation-based functional ProteoForm assessment in bottom-up proteomics data.

Methods: COPF leverages the concept of peptide correlation analysis to systematically assign peptides to co-varying proteoform groups. Conceptually the COPF workflow can be divided into four steps: First, the intensities of peptides assigned to the same gene are determined from the corresponding MS signals across all measured samples. Second, all pairwise peptide correlations within a protein are calculated. Third, the correlation distance is used for hierarchical peptide clustering into proteoform groups. Finally, proteoform scores and corresponding p-values are calculated based on the within- vs. across-cluster correlation.

Results: We first benchmark COPF against state-of-the-art software, demonstrating its unique applicability to complex experimental designs, the possibility to detect proteoforms that cover large sequence stretches, and the availability of an accurate error model. We next apply COPF to protein complex co-fractionation data where cells in two cell cycle stages are compared. Our results indicate that COPF is capable to systematically detect assembly- and cell cycle-specific proteoform groups. As a second example, we apply COPF to assign functional proteoform groups in a typical bottom-up proteomic cohort study consisting of five tissue samples from the mouse BXD genetic reference panel. In this dataset, COPF could determine several tissue-specific proteoform groups. Our examples overall include proteoform groups generated by proteolytic cleavage, alternative splicing, and multiple phosphorylations.

Conclusions: We envision that COPF can make a significant contribution towards the systematic assessment of proteoform groups across large bottom-up proteomic datasets, thereby opening new avenues for linking proteoforms to biological function.

Poster Abstracts (P)

P01.01

Methylproteome and Phosphoproteome Crosstalk in the Maintenance and Differentiation of Glioma Cancer Stem Cells

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Introduction
Methylation of proteins, mainly on lysine and arginine, can modulate their structure, function and stability. In part this is achieved through crosstalk with other post-translational modifications including acetylation and phosphorylation. Aberrant methylations and phosphorylations are frequently reported in cancers, but how crosstalk relates to mechanisms observed in the process of carcinogenesis is poorly defined. In this study glioma initiating cells (GICs) were used as a model in which to investigate differences in the crosstalk between the methylproteome and phosphoproteome in the processes of cancer stemness compared to differentiation.

Methods
Glioma cells were cultured as both spheroids and differentiated forms for up to 72 hours. Cells were harvested at 0h, 24h and 48h with three biological replicates. These were lysed and phosphopeptide enrichment was performed using HAMMOC. Peptides were analyzed by the LFQ mode using an Easy nanoLC-Orbitrap Fusion Tribrid system (Thermo Fisher Scientific) equipped with 75 um x 12 cm C18 3um Nikkyo-RP-nano Column (Nikkyo Techonos), and phospho-peptides identification/quantitation and PTM site analyses were performed on PD 2.4.

Results
A total of 9937 proteins (4143 protein groups) were identified through 23,046 peptide groups. 104 peptides were identified which were both methylated and phosphorylated in 74 proteins. Most of these 74 proteins are involved in transcription and cell morphology. In all the identified peptides the distance between methylation and phosphorylation was 2 to 15 residues. Of the identified peptides, 43 could not be fully quantified. Of the quantifiable peptides the two most interesting candidates were Histone 3.1 and Isoform A1-A of Heterogeneous nuclear ribonucleoprotein A1 in which the peptide with both PTMs together was only found in differentiated cells.

Conclusions
The understanding of methylation and phosphorylation crosstalk in GIC stemness maintenance and differentiation will be useful not only in elucidating therapy resistance mechanisms but also in identifying potential therapeutic targets.

P01.02

Cellular Protein Perturbations Identify Toxicity Pathways Associated with ZnO Nanoform Exposures

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Introduction: Zinc oxide nanoforms belong to the metal oxide family of engineered nanomaterials. Their attractive electrical, optical, magnetic and catalytic properties in the nano size compared to the bulk form have led to the enhanced production and utility in various applications, consumer products such as building materials, sunscreens, moisturizers, food packaging and in biomedical field. This has led to concerns of exposure to ZnO nanoforms and subsequent impact on environmental and health effects. Although studies are being conducted on toxicity of nano-sized ZnO, toxicity information on the various nanoforms of the same chemical is scarce. Methods: In this work, a set of well-characterized ZnO nanoparticles (NPs) of varying sizes and surface-modifications (e.g uncoated 30, 45, 53 nm; coated with silicon oil, stearic acid and triethoxysilane derivatives) were therefore screened for in vitro cytotoxicity in two cell types, namely, human lung epithelial cells (A549) and mouse monocyte /macrophage (J774) cells. ZnO (bulk) and ZnCl2 were used as reference particles. Cytotoxicity was examined 24 h post exposure by assessment of CTB (cell viability), ATP (cellular energy metabolism) and %LDH released (cell membrane integrity). Secreted and cellular proteins were analyzed by multiplexed protein array analysis and LC-orbitrap MS analysis. Results: Exposure- and cell type-specific cytotoxic and protein responses were observed. Relative potencies of NPs were influenced by physicochemical properties (e.g. surface area, agglomeration, metal content). Cellular protein changes revealed NP exposure-related differential activation of signalling pathways relevant to inflammation and cell injury. Biological processes affected by ZnO NPs included apoptosis, necrosis and production of reactive oxygen species. Conclusions: Our findings demonstrated that application of proteomic analysis can be valuable in understanding nanomaterial exposure-related toxicity mechanisms and support risk analysis of these ZnO nanoforms, and also can inform on the design & selection of safer nanomaterials.

P01.03

Future Perspectives and Strategies for Data-Independent Acquisition on Orbitrap MS Instruments

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Introduction
Biological samples frequently generate a complex analytical matrix. Despite the recent innovative DIA-MS approaches in fast-scanning MS such as Q-TOF, the Orbitrap MS is most widely used and provides ideal mass resolution. Due to the limit of scanning speed, the current DIA methods still have to use rather large isolation windows, resulting in multiplexed MS2 spectra that cannot be directly analyzed by shotgun database searching engines. On the other hand, the bottom-up proteomics has common experimental features, e.g. the similar peptide length, sequential elution of the peptides along LC separation, and predictable mass-to-charge (m/z) difference between peptides and their post-translational modification (PTM) forms.

Methods
Based on the above common features, we devised two strategies to allocate small DIA windows intelligently. The first method uniquely schedules the small isolation windows (as small as ~5 m/z) in different retention time blocks, by taking advantage of the fact that larger peptides are normally eluting later from LC. The second method incorporates the multiplexity of BoxCar MS1 acquisition via repeated sample injections and non-consecutive, small DIA windows (~2.5 m/z), so that every peptide and most of their PTMs are analyzed in different MS2 scans.

Results
We were able to use the shotgun database searching tools to directly analyze the DIA-MS data of intelligent allocation schema. We found our new 4-hour DIA-MS consistently quantified ~8,200 protein groups in cell line samples, with a much greater signal-to-noise ratio and thus a better relative quantification accuracy in both label-free and SILAC experiments compared to conventional DIA-MS.

Conclusions
Given the above results, we will try to address e.g., how to envision the future DIA-MS on Orbitrap? How would the future DDA and DIA workflows converge on faster MS analyzers? Could we adjust current DIA-MS acquisition and data strategies before the “ideal” instrument shows up?

P01.04

Progress Identifying and Analyzing the Human Proteome: 2021 Metrics from the HUPO Human Proteome Project

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Introduction: The Human Proteome Project (HPP) is the flagship initiative of HUPO. Its goals are a complete parts list and integration into major multi-omics studies.

Methods: The HPP consists of Chromosome-centric, Biology and Disease-driven, and Resource Pillar teams (48 in all). PeptideAtlas, MassIVE, and neXtProt reanalyze and curate data from the entire community.

Results: The 2021 HPP Metrics show protein expression now credibly detected (neXtProt PE1 level) for 18,357 (92.8%) of the 19,778 predicted proteins coded in the human genome, up 483 since 2020 from reports throughout the world; 17,100 PE1 proteins are identified by mass spectrometry and 1257 by other methods. Conversely, neXtProt PE2, PE3, and PE4 missing proteins (MP) have been reduced by 478 to 1421. This represents remarkable progress on the proteome parts list.

Utilization of proteomics in biological and clinical studies continues to expand with many important findings and effective integration with other omics platforms. We present highlights from the Immunopeptidomics, Glyco-Proteomics, Infectious Disease, Cardiovascular, Musculo-Skeletal, Liver, and Cancers B/D-HPP teams and Knowledgebase, Mass Spectrometry, Antibody Profiling, and Pathology resource pillars. Diverse studies of SARS-CoV-2 viruses and infections, proteogenomics to guide immunotherapy and chemotherapy of cancers, protein-protein interactions and post-translational modifications in host-infectious agent pathogenesis, integration of glycosylation sites from Glyconnect into neXtProt, altered sarcomeric proteoforms in hypertrophic cardiomyopathy, initiation of Extracellular Vesicles and PhosphoPeptide Atlases, and the Single Cell Type Human Protein Atlas are notable advances.

Conclusions: As clinical importance of proteomics grows, based on mass spectrometry, aptamers, and Olink antibodies, it is timely to consider the ethical dimensions important for informed consent, protection of privacy, respect for diversity, and report and explanation of actionable incidental findings.

P01.05

A TMTpro 18plex Proteomics Standard for Assessing Protein Measurement Accuracy and Precision

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Introduction
Multiplexed quantitation strategies using Thermo Scientific™ Tandem Mass Tags™ (TMT™) enable precise measurement of peptide or protein abundance from multiple samples using a single high-resolution LC-MS analysis. However, co-isolation of peptides with similar mass-to-charge can suppress protein abundance ratios resulting in less accurate measurements. Our previously launched TMT11plex standard is useful for method optimization and assessing instrument performance. However, it does not enable accurate measurements of the knockout protein abundances. Here, we describe new prototype standards using TMTpro 18plex reagents capable of assessing both accuracy and precision of multiplex protein quantitation.

Methods
To generate different prototype standards, a parental Saccharomyces cerevisiae strain (BY4741) was mixed with different fixed amounts of one or more knockout strains (Met6, His4, or Ura2). Standard sample mixtures were labeled using TMTpro 18plex reagents and combined to measure the accuracy and precision of TMTpro quantitation. We performed LC-MS/MS analysis using an Thermo Scientific™ Ultimate™ 3000 nanoLC coupled to Thermo Scientific™ Eclipse Orbitrap™ or Exploris™ 480 mass spectrometers, with or without a FAIMS Pro™ Interface. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 3.0 software using the SEQUEST® HT and COMET search engines.

Results
To assess the different standard mixing schemes, parental yeast extracts were mixed using one or more knockout strain extracts and labeled using TMTpro 18plex reagents in duplicate or triplicate. These standards enabled assessment of interference on protein abundance measurements at different sample loads, LC-MS gradients, MS methods, and platforms. As expected, real time search (RTS) synchronous precursor selection (SPS)-based methods provided the best accuracy and precision as compared to MS2 methods. The use of a FAIMS Pro Interface also improved the accuracy of the protein measurements for MS2 and MS3 methods.

Conclusions
TMTpro 18plex yeast standards enables the assessment and optimization of instrument performance for TMTpro isobaric tag-labeled, multiplex samples.

P02.01

Evaluation of Long Nanoflow Columns with Core-Shell Based Chromatographic Phases in Data Dependent Acquisition Workflows

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Introduction: One of the main acquisition workflows for discovery proteomics is data dependent acquisition (DDA) for the identification of large numbers of peptides. Nanoflow chromatography is often used in DDA workflows in order to obtain the highest sensitivity on digested samples. Peak shape and resolution are critical to allow the MS system to sample as many peptides as possible. Here, new long nanoflow columns packed with core-shell chromatographic phase were evaluated for the impact on the protein identification workflows.

Methods: In the current study, three different nanoflow column lengths (15, 25, and 50 cm) were packed with normal porous C18 phase and also core-shell Kinetex C18 phase (both 2.6 µm and 5.0 µm particle sizes). Several gradient durations ranging from 60 to 180 minutes were tested across the column types for DDA workflows. K562 and HeLa cell digests were evaluated at several loads and a trap-elute workflow was implemented with an analytical flow rate of 300 nL/min. The TripleTOF 6600+ system and ZenoTOF 7600 system was used for all data acquisition and data was processed using the ProteinPilot App in the cloud.

Results: Here, the impacts of column phase, column length and gradient duration on protein and peptide identifications were assessed. As expected, protein and peptide identifications increased as the gradient length increased and column length increased, with optimal identifications observed with a 180 min gradient and 50 cm column length. Interestingly the core-shell packed columns provided more protein and peptide identifications at all gradient lengths, with average increases in protein IDs of 10-20% depending on column length. Next the impact of higher sensitivity MS/MS (Zeno MS/MS) on protein ID gains will be evaluated using the optimized long column conditions.

Conclusions: Using a 50 cm nanoflow columns packed with core-shell chromatographic phase improved protein and peptide identifications in DDA workflows.

P02.04

Real-Time Search Assisted Acquisition on a Tribrid Mass Spectrometer Improves Coverage of Multiplexed Single-Cell Proteomics

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A recent major breakthrough in single-cell proteomics (scMS) was the introduction of the SCoPE method, where isobaric labeling is used to multiplex single-cells together with a carrier channel to be then measured in a single LC-MS run. The Orbitrap Eclipse introduced real-time search (RTS) assisted acquisition, improving proteome coverage in synchronous precursor selection (SPS)-MS3 acquisition of TMT labeled samples. In this work, we compared the performance of multiplexed scMS using MS2 acquisition against RTS-SPS-MS3. Furthermore, we present a new acquisition strategy (termed RETICLE) that utilizes RTS for quantitative MS2 acquisition and show that this strategy outperforms MS2 and RTS-SPS-MS3 in multiplexed scMS applications.

To benchmark the quantitative performance of the different acquisition strategies, we used the OCI-AML8227 culture model. This model maintains the hierarchical nature of Acute Myeloid Leukemia (AML), and consists of at least three distinct cell differentiation stages with differences on proteome level detectable by scMS. We benchmarked the three acquisition strategies (MS2, RTS-MS3, RETICLE) by using not only a diluted standard from the OCI-AML8227 culture model, but also by measuring 6 real scMS datasets with over 100 single cells each. Results were compared regarding proteome depth and quantification performance.

Analysis of the raw files showed that with RTS-MS3 and RETICLE, RTS-triggered scans have considerably higher identification rates (RTS-MS3=60%, RETICLE=80%) compared to standard MS2 (40%), resulting in over 20% more proteins being quantified in the single-cell channels. We found that increased ion injection times overall improved the separation, exemplifying the importance of sampling enough ions for accurate quantification. Furthermore, the RETICLE data showed the best separation of differentiation stages, even better than RTS-MS3, indicating that the sensitivity loss in MS3 outweighs the gained quantitative accuracy. In conclusion, RETICLE enables higher scMS proteome coverage compared to MS2 and RTS-MS3 acquisition methods, while also providing improved quantitative performance and sensitivity.

P02.05

A Highly Efficient and Automated Workflow for Label-Free and Multiplexed Single Cell Proteomics

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Introduction: Single-cell technologies have shaped modern molecular biology, yet accurate global proteome measurements at such resolution is still challenging. Recently dedicated sample processing in conjunction with latest generation chromatographic separation and MS-instrumentation improved coverage. Here we present an automated workflow for both label-free and multiplexed single cell proteomics (SCP) at unprecedented sensitivity.

Methods: Cells were dispensed in 40-100nL master-mix (0.2% DDM, 20ng/µL trypsin) for lysis and digestion. Multiplexed samples were labeled with 22mM TMT, quenched (hydroxylamine and HCl), and pooled via centrifugation. Samples were acquired with the Waters nanoEase m/z BEH C18-analytical column on an Orbitrap ExplorisTM 480 with FAIMS Pro.

Results: Our dedicated substrate, the proteoCHIP allows simultaneous processing of up to 192 cells within twelve sets of 16 multiplexed single cells. The workflow is performed entirely within the cellenONE®, a platform combining single cell isolation and nanoliter reagent dispensing. The temperature and humidity controlled cellenONE® circumvents evaporation and even allowed lowering the initial reaction volume 2.5-fold, which dramatically reduced background signal and increased protein identifications by 25%. This, in conjunction with our tailored label-free single cell workflow reproducibly yields 500 proteins. Samples are pooled via centrifugation to the proteoCHIP funnel, which is directly interfaced with standard autosamplers via a custom adaptor to eliminate all sample transfer. This allowed for cell-type dependent separation of 50 multiplexed HeLa and HEK cells based on close to 2,000 proteins with a median reporter ion S/N value of 255. Additionally, the analysis of in-vitro derived cardiac organoids corroborated successful co-differentiation and cell specification into early cardiomyocyte and endothelial lineages but revealed a highly similar and yet undifferentiated 'base-proteome'.

Conclusion: The proteoCHIP workflow in conjunction with the cellenONE® outperforms reporter ion S/N of previous SCP by nearly 18-fold, enables sensitive cell type dependent clustering across multiple analytical runs and defines early cardiac lineages.

P02.06

Digital Microfluidics for Proteomics Analysis of Few or Single Mammalian Cells

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Introduction
Recently, novel sample preparation sample preparation methods have enabled the LC-MS/MS-based, label-free proteomics analysis of few or even single mammalian cells. However, these methods often require costly nanodispensing technologies. We here present digital microfluidics (DMF) as an emerging and readily affordable droplet handling platform, allowing the preparation of ready-to-analyze proteomics samples in the range of 100 down to single mammalian cells. Further, we developed methods for on-chip quantitative isobaric labeling of peptides derived from minute cellular samples. Additionally, we show the potential of DMF for functional nanoproteomics, by employing functionalized magnetic beads in DMF sample preparation.

Methods
All experiments were performed on a DropBot DMF system (Sci-Bots Inc.). On-chip DMF protein clean-up was achieved by single-pot, solid-phase-enhanced sample preparation (SP3), facilitated by means of a magnetic lens setup. For analysis of few or single Jurkat T cells, cells were dispensed via FACS onto opened chip arrays positioned in a 3D-printed holder. Cells were lysed by rapid solvent-SP3, and digested samples were directly loaded onto the analytical LC column via a semi-automated chip-to-autosampler interface, constructed from low-cost and 3D-printed components.

Results & Discussion
We successfully optimized the composition of buffers and detergents, which enabled facile DMF droplet movement and the label-free identification of, e.g., up to 1,200 proteins from approximately 100 cells. For quantitative proteomics analysis, we performed isobaric labeling in the presence of a LC-MS compatible maltoside detergent. Analyzing 75 Jurkat T cells treated with an anti-cancer drug, we identified 39 differentially abundant proteins. Lastly, for the first time, we show a label-free single cell proteomics workflow based on the SP3 magnetic bead protocol, using a rapid solvent lysis and direct chip-LC coupling.

Conclusions
DMF-SP3 holds great potential for functional nanoproteomics.

P02.07

Streamlined Single-Cell Proteomics by the All-in-One Chip and Data-Independent Acquisition Mass Spectrometry

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Introduction
Single-cell proteomics provides the ultimate resolution to reveal cellular phenotypic heterogeneity and functional network underlying biological processes, yet its implementation in a miniaturized and streamlined device is rather underexplored. We describe herein a new workflow combining an integrated chip and data-independent acquisition mass spectrometry (DIA-MS) for sensitive microproteomics analysis down to single cell level.
Methods
This study developed a highly integrated device (iProChip) coupled with DIA-MS for single cell proteomics analysis. The iProChip was designed as an all-in-one proteomic station, offering built-in features including quantifiable cell capture, imaging and lysis, protein digestion, and peptide cleanup. Furthermore, the DIA-MS was found to offer improved proteome coverage than the data-dependent acquisition mode by 2.3 fold for profiling 1-100 cells.
Results
The iProChip-DIA workflow successfully characterized ~1000 protein groups from individual cells with 1% FDR. The versatility of iProChip-DIA was demonstrated using both the human adenocarcinoma (PC-9) and chronic B cell leukemia cell (MEC-1) respectively. The results revealed good performance of 5 orders of proteome coverage, >100-fold quantification range, high reproducibility and low between-run missing values. Furthermore, at the level of 1-100 cells, important drug targets and biomarkers associated with lung cancer and B-cell receptor pathway were readily quantified, suggesting the potential utility of the developed approach for translational applications.
Conclusion
The iProChip is versatile and scalable to accommodate existing proteomic methods to achieve sensitive and multiplexed quantitative proteomics profiling under desired context. Such strategy is expected to find applications for limited input samples, e.g. rare cell population from clinical specimens. The presented approach might open a new avenue for bringing distinct functionalities into a single miniaturized platform and enable the proteomics analysis at single cell level.

P02.08

Comparison of Epithelial and Stromal Proteomes from Colorectal Adenoma to Carcinoma

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Introduction: It is well accepted that tumor microenvironment (TME) of colorectal cancer (CRC) plays a key regulator to CRC development and metastasis. Most cases of CRC occur malignancy of epithelial cells and undergo a process from normal, adenoma to carcinoma (N/A/C). There are many reports to explore the molecular mechanism of the CRC N/A/C process, however, is a few of study regarding the pathological changes of stroma during the process. Herein, in this study, we explored microenvironment change during N/A/C sequence using proteomics strategy.
Methods: Surgery tissues were carefully excised by laser capture microdissection (LCM) from 17 CRC patients for protein extraction. The proteomic analysis of both bulk and LCM samples were quantified with DIA approach. Bioinformatic analysis was performed in R.
Results: Proteomic landscapes in epithelia were quite difference in all the stages of CRC, whereas that in stroma were roughly divided to two patterns, in which the stromal proteomes in adenoma were comparable with that in carcinoma, and the adjacent proteomes were very different from that in adenoma and carcinoma. Compared of the estimation results in adjacent, the immune cells such as CD4 T cell, CD8 T cell and dendritic cell in the tissues of adenoma/carcinoma stroma were significantly increased, indicating a strong immune response happened in disease microenvironment. Pathway enrichment showed that pathway of antigen presentation in the tumor and stromal regions were overall assessed. The relevant assessment revealed that antigen presentation appeared overall activation, however, such presentation was attenuated in the tumor regions.
Conclusions: Based on LCM&DIA, the microenvironment of CRC can be clearly characterized. Although a traditional view regarding the microenvironment changes from N/A/C is hypothesized as stage-dependent, the proteomics endorse another postulation that the microenvironment of adenoma and carcinoma shared some molecular similarity.

P03.01

Defining Mechanisms Underlying Virus Regulation of Mitochondrial Bioenergetics During Infection

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Introduction: Alterations of mitochondrial functions and cellular metabolism are hallmarks of nearly all virus infections. As obligate intracellular parasites, viruses rely on mitochondria for the production of biosynthetic precursors and energy necessary for generating new viral particles. The prevalent pathogen human cytomegalovirus (HCMV) alters both mitochondrial structure and metabolism during its replication. However, how HCMV upregulates mitochondrial bioenergetics remains unknown.
Methods: Employing a multidisciplinary approach integrating proteome and interactome datasets with super-resolution confocal microscopy, LC-MS based metabolite profiling and metabolic assays, we identify a previously uncharacterized viral protein, pUL13, which targets the mitochondria and increases oxidative phosphorylation during infection.
Results: We use targeted mass spectrometry analysis of the HCMV proteome during infection, coupled with molecular virology techniques to establish that pUL13 is required for productive HCMV replication. We then quantify temporal cellular proteome changes during infection and demonstrate that pUL13 alters electron transport chain (ETC) protein abundances. Using LC-MS based metabolite profiling and live-cell Seahorse metabolic assays to monitor cellular respiration, we establish pUL13 as necessary and sufficient to increase cellular respiration, not requiring the presence of other viral proteins. To mechanistically define the function of pUL13 in regulating cellular respiration, we characterize the spatio-temporal pUL13 functional interaction network during infection. We discover and validate that pUL13 targets the MICOS complex, a critical regulator of mitochondrial architecture and ETC function. We then use stimulated emission depletion (STED) super-resolution microscopy analysis to visualize the impact of pUL13 on mitochondrial ultrastructure.
Conclusions: Our findings address the outstanding question of how HCMV modulates mitochondria to increase bioenergetic output and expands the knowledge of the intricate connection between mitochondrial architecture and ETC function. Importantly, this is the first known instance of a virus protein targeting the MICOS complex to increase bioenergetic output, highlighting a mechanism that other virus pathogens might also possess.

P03.02

Cell-Surface Proteomics: Novel Methodology for Identifying Cell-Surface Proteins of Toxic Dinoflagellates

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1. Introduction

Toxic dinoflagellates are the major causative agent that produces paralytic shellfish toxins (PST) and inflict serious threat to human health and global food safety. Thus, identification of toxic dinoflagellates has become an essential topic in recent marine research. Several existing methods were developed to enrich proteins in thecal plates for better identifications of surface proteins, such as cold-shock-induced cell wall isolation. However, these methods failed to distinguish surface proteins of which exposed to the extracellular environment. Moreover, yields of surface and thecal proteins were low and may be easily contaminated with intracellular proteins during lysis of the dinoflagellates. In response to these challenges, this study aimed to establish an advanced methodology for enriching the surface proteomes of the harmful species. In this study, five toxic dinoflagellates, Alexandrium minutum, A. lusitanicum, A. tamarense, Gymnodinium catenatum and Karenia mikimotoi, were selected.

2. Methods

With the aid of a plasma-membrane-impermeable labeling agent (sulfo-NHS-ester), intact-cell labelling on the cell surface of the dinoflagellates was facilitated. After that, a novel surface protein extraction method was demonstrated to enrich surface proteins and those that are facing the extracellular space. The protein extracts were analyzed by liquid chromatography linked-tandem mass spectrometry (LC-Orbitrap-MS/MS) together with bioinformatic search of in-house transcriptomic databases of these five species constructed. Surface proteomes of these species were then generated.

3. Results

With the novel technique, 16 extracellular-facing transport proteins, 11 surficial species-specific proteins and 5 thecate-species-specific proteins were successfully identified. Extracellular-exposing regions of these proteins were also determined.

4. Conclusions

These findings indicated the compatibility and improvement of this methodology to identify and locate the cell-surface proteins in dinoflagellates. It would contribute to the outlining of outermost cellular structures in dinoflagellates as well as the future development of techniques for differentiating dinoflagellates with similar morphologies.

P03.03

The Human Fallopian Tube Proteome

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Introduction
The fallopian tubes (FT), or oviducts, are ciliated tubular seromuscular organs that connect the ovaries to the uterus. The FTs facilitate the movement of gametes and the fertilized egg, and provide an optimal environment for successful fertilization. Many of the proteins involved in these processes are however uncharacterized, and a comprehensive description of the FT proteome is yet to be published. The aim of the study was to integrate transcriptomics with antibody-based proteomics and utilize the Human Protein Atlas (HPA) infrastructure to further map the spatial cell type-specific expression of the complete set of proteins elevated in the FT.

Methods
The analysis is based on bulk and single-cell RNA sequencing (scRNAseq) data gathered through HPA and external sources, combined with immunohistochemistry (IHC) on a custom made tissue microarray containing both FT and other ciliated tissues. In an attempt to emphasize protein-coding genes that are highly relevant for FT function, the study focused on 312 genes that had an elevated mRNA expression in the FT (FT-elevated) compared to other tissues and organs.

Results
Hierarchical cluster analysis of the 312 FT-elevated genes identified testis as the organ with the most similar mRNA expression compared to FT, followed by brain, epididymis, ovary and lung. IHC and scRNAseq analysis identified motile cilia as a common denominator among the correlated organs. Among the genes validated with IHC (n=128), the vast majority were localized to different parts of motile cilia.

Conclusions
Through an exploration of 312 genes with elevated expression in FT, based on an integrated omics approach, a substantial portion of corresponding proteins were identified as components of macrostructures of motile cilia. These findings thus contribute to further understanding of proteins not previously mapped in the context of motile cilia or FT, possibly essential for the function of the FTs in human reproduction.

P03.04

Identification of Cell Type-Specific Endometrial Markers through Integration of Single-Cell Transcriptomics and Spatial Proteomics

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Introduction

The endometrium undergoes hormonal-dependent alterations across the menstrual cycle, in which menstruation is followed by a proliferative and a secretory phase separated by ovulation. There is a great need for increased knowledge about physiological differences in the endometrial tissue at these different hormonal stages, and to which extent these differences can be explained by proteomic changes. The aim was therefore to obtain a list of 100 cell type- and menstrual phase-specific endometrial markers.

Methods

Publicly available single-cell RNA sequencing (scRNAseq) data of human endometrium from the GEO database (GSE111976) and the Reproductive Cell Atlas, were reanalyzed using Seurat V4.0 in R (CRAN). Marker lists were obtained for each dataset per cell type, and included genes filtered based on antibody reliability data from the Human Protein Atlas (HPA) database. The genes were further selected based on differential expression analysis with an average expression two times higher for each cell type. This procedure was also undertaken for the different menstrual phases. The marker lists were further screened for distinct immunohistochemistry profiles in the HPA database where the cell type-specificity in the respective endometrial cell type could be confirmed.

Results

The reanalysis of scRNAseq data resulted in cell type-specific marker lists from both GSE11197 (n=356) and Reproductive Cell Atlas (n=642), and menstrual phase specific marker lists for epithelia (n=226) and stroma (n=138). The manual screening of the lists resulted in a top 100 marker list; including proteins such as PEAP (epithelia), TPPP3 (ciliated epithelia), GJA1 (stroma), CNN1 (smooth muscle), CD93 (endothelia), CD247 (lymphocytes), and CD68 (macrophages).

Conclusions

Integration of scRNAseq- and spatial protein data resulted in a top 100 list of cell type- and menstrual phase-specific endometrial markers. These will be used for future analyses; including co-localization approaches using multiplexed immunofluorescence techniques to better understand spatial changes throughout the menstrual cycle.

P03.06

System Wide Profiling of Protein Interaction Dynamics Links Host Innate Immunity and DNA Damage Responses

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Rapid and dynamically shifting protein-protein interactions are central to sensing viral infections and mounting antiviral immune responses. In response, viruses have developed strategies to suppress or hijack immune response proteins, which underscores the biological complexity of host-pathogen interactions during immunity. Accordingly, conventional approaches to study the protein-protein interactions in the context of innate immune sensing fail to capture the global scope and dynamic behavior of protein complexes as they alter through space and time during a viral infection. We have applied and advanced the Thermal Proteome Profiling Mass Spectrometry (TPP-MS) method to characterize construct a cell-wide portrait of protein complex dynamics that coordinate the innate immune response to Herpes Simplex Virus 1 (HSV-1) infection in primary human fibroblast. By leveraging TPP-MS to infer and track protein interactions we identified that IFI16, a nuclear DNA sensor that serves as a central platform for HSV-1 immune responses, coordinates with the cellular DNA damage response. Our TPP-MS analysis, along with high-resolution microscopy and molecular virology, links IFI16 sensing of viral DNA in the nuclear periphery to the master DNA damage response (DDR) regulatory kinase, DNA-PK—which was necessary for the antiviral and inflammatory response to infection. Finally, phospho-peptide enrichment of DNA-PK substrates uncovered that IFI16 modified by the DDR kinase after both DNA damage and viral infection, which regulates IFI16-driven cytokine responses. Our study represents the first cell-wide characterization of PPIs during HSV-1 infection, and further uncovers a missing link in the immune signaling pathway that places IFI16 and DNA-PK central to herpesvirus innate immunity.

P03.07

Spatial Proteomics Analysis of Ovaries from Women in Reproductive Age

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Introduction
The ovary is an extremely dynamic organ, which has a crucial role in both endocrine and reproductive systems. Monthly, it undergoes structural changes to release oocytes during reproductive years (from puberty to menopause). This leads to a high degree of variation in gene expression during menstrual cycle and throughout lifespan. The Human Protein Atlas (HPA) aims to compile information regarding expression profiles in human organs on both RNA and protein level. Thus, 173 genes were shown to have elevated expression in ovary. There is, however, no information comparing expression during reproductive and post-menopausal years. Our aim was to build a framework to identify proteins potentially involved in reproductive function of the ovary.

Methods
RNA sequencing data from the GTEx project for ovary was re-analyzed to identify genes differentially expressed according to age, potentially corresponding to markers related to reproductive function. The results were validated with immunohistochemistry (IHC) using a unique tissue microarray for ovaries including both pre- and post-menopausal tissues. We additionally used a multiplexed immunofluorescence approach for selected candidates to map the details of the follicular structure.

Results
Gene expression in ovaries of women in reproductive age (n=26) was compared with women in post-menopausal age (n=36), and 509 genes had at least two-fold higher mean value of RNA expression in the reproductive age group. Among these, 174 had reliable antibodies available within the HPA project, and 14 (ZP4, ZP2, FIGLA, ZP3, STAG3, DSP, CDH2, ALOX15B, SPP1, HMGB3, ELAVL2, INSL3, RTL9, DDAH1) were selected for IHC staining.

Conclusions
This preliminary study allowed us to identify well-known oocyte markers (ZP2), and provided us with new insights regarding the spatial localization of proteins with unknown function in ovary (SPP1). These findings constitute a first step towards the complete exploration of the spatial proteome of the ovary throughout the lifespan of women.

P03.08

Differential Regulation of Promyelocytic Leukemia-Nuclear Body (PML-NB) Proteins during Oncogene Induced Senescence

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Somatic cells accumulate several mutations during an organism's life span. Cells, however, have developed intrinsic mechanisms to prevent tumorigenesis upon deleterious mutations, most notably, oncogene-induced senescence (OIS). OIS is a process in which cells enter a state of permanent cell cycle arrest in response to the activation of proto-oncogenes, such as RasG12V. The molecular mechanisms underlying OIS are complex, and remain to be characterized. Using IMR90 human diploid fibroblasts (ATCC CCL-186) as a model, we applied quantitative proteomics and phosphoproteomics to better understand the regulation of OIS, which can be crucial for the prevention of tumor formation. IMR90 cells were transduced with the inducible protein ER:RasG12V lentivirus and OIS was induced by treating cells with 100nM (Z)-4-Hydroxytamoxifen (4-OHT) for 0, 2, 4 or 6 days. Purified nuclear proteins were digested with Trypsin and peptides were desalted using C18 spin columns before the enrichment of phosphopeptides using the PolyMaC spin tips. (Phospho)peptides were then separated with a reverse phase column, and analyzed with the Orbitrap Fusion Lumos mass spectrometer. Data was analyzed with the MaxQuant and Perseus software. Nuclear proteome of cells undergoing OIS revealed a total of 6264 proteins, from which 1904 proteins were significantly regulated ($p<0.01$). Our dataset also contained 59 significantly regulated PML-nuclear body proteins, and 103 PML-NB phosphoproteins, at day 6 of 4-OHT treatment , including TRIM family proteins, transcription factors such as Stat3 and histones H3-4 and H2AX . This constitutes the largest number of identified putative PML interacting proteins in a single LC-MS experiment, and the largest reported number of PML-NB proteins that are regulated upon OIS. Interestingly, PML-NB were mostly downregulated during the progression of OIS. Overall, our data provides preliminary evidence that PML-NB differentially regulated during OIS, in which PML-NB potentially serves as a hub for protein degradation, and, thus, modulates OIS.

P03.09

Combining SDS with Subcritical for Continuous Flowthrough Extraction of Proteins from Food Samples.

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Background: Subcritical water (SW) is defined as water at temperatures above 100 oC but below the critical point (374°C). 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. 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: yeast, flaxseeds, and rice bran were subjected to extraction by injecting suspended samples into a customized extractor. The device consists of an HPLC pump, high-pressure injector, stainless steel tubing wrapped around a programmable heater, followed by a heat sink, inline filter, and restrictor lines to maintain high pressure within the system. The device maintains SW at temperatures up to 200oC. Protein recovery was determined by BCA assay, with SDS PAGE used to monitor protein degradation. In-gel digestion and bottom-up proteomics analysis with LC-MS/MS were utilized to classify the extracted proteins and further assess the integrity of the extracted protein.
Results: The extraction efficiency of yeast proteins in SW is significantly enhanced with the addition of SDS up to 5%. The optimal temperature for SW extraction was 105-150 oC. SDS PAGE analysis revealed distinct protein bands over a range of molecular weights, suggesting that intact proteins are preserved through the SW extraction process. Mass spec results showed similar MW protein distributions observed between temperature of 90 oC up to 150 oC, however, distribution of MW changed at 195 oC suggesting protein degradation at 195 oC. Intracellular mitochondrial proteins were found at 195 oC and were absent at 90 oC.
Conclusion: The combination of SDS with subcritical water enhances the extraction efficiency of proteins in yeast. The optimal temperature of extraction is dependent on targeted proteins.

P03.10

Impacts of Intracellular-Advanced Glycation End Products in Pancreatic Ductal Epithelial Cells

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Introduction:
Glycation is a non-enzymatic irreversible reaction between reducing sugars and proteins resulting in advanced glycation products (AGEs). Several physiological and social factors such as diabetes, unhealthy diet styles, cigarette smoking have been known to promote AGEs accumulation in the body. Currently, there is an increasing attention towards AGEs on different diseases including cancer due to the impact from AGEs on physiological functions.

Method:
Normal and malignant pancreatic ductal epithelial cells were treated with different concentrations of glyceraldehyde (GA) to induce intracellular-AGEs, and cell lysates were collected for analyzing proteomic changes. Briefly, proteins were harvested from cells with lysis buffer and subjected to reduction and alkylation followed by tri-carboxylic acid protein purification. The samples were tryptic digested, and the peptides were analyzed by LC MS/MS. Raw data were processed and searched against Uniprot Human Database using Proteome Discover. Label-free quantification was carried out to identify the dysregulated proteins (fold change > 1.5, P< 0.05) associated with GA treatment. Western blot was performed to determine the autophagy in these cells.

Results:
Our study indicated that GA treatment increased the intracellular-AGE accumulation, in which more glycations occurred on lysine residues compared to arginine. The accumulation of AGEs altered the biological processes by downregulating the metabolic protein networks. AGE formation also decreased protein acetylation, which may contribute to the dysregulation of a wide range of cellular functions associated with cellular homeostasis. Autophagy was up-regulated in these cells at higher concentrations of the GA treatment and E3 ubiquitin ligase expression was decreased during AGE accumulation. Decreased ubiquitination and increased autophagy implicated that cells might utilize non-canonical pathways for cell-regeneration, which is predominantly seen in cancer.

Conclusion:
These findings suggested that the formation and accumulation of intracellular-AGEs altered the cellular physiology and had a profound impact on cellular processes related to acetylation and ubiquitination.

P03.11

Streamlined Use of Protein Structures and Virtual Reality to Analyse Variants

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Introduction

A core task in bioinformatics is assessing the impact of missense variants on a protein's function. Mapping variants onto available 3D structural models can reveal spatial context that provides insight into a variant's functional impact. While many available tools enable variants to be mapped onto structures, using these tools can often be difficult. Additional problems arise with large and complex structures, which can be intrinsically difficult to navigate; this may be improved by using extended reality (XR) methods, which better engage perceptual and cognitive processing compared to conventional displays. Unfortunately, very few variant mapping tools currently support XR.

Methods

To address these limitations, we implemented a set of XR and variant mapping capabilities. These were integrated into Aquaria (O'Donoghue et al. Nature Methods, 2015), a web-based molecular graphics system that provides a total of >100 million protein structure models, pre-calculated using the ~500,000 sequences in SwissProt.

Results

The new version of Aquaria (https://aquaria.app) allows a variant to be mapped onto any related 3D structure via an URL (e.g., https://aquaria.app/Human/WT1?Arg370Leu). In the resulting webpage, the spatial context of a specified variant can be easily examined using any of the (on average) ~200 related structural models available for each protein. Exploration using XR is supported on most smartphones, and on Windows Mixed Reality devices such as HoloLens. Besides variants, protein structures can also be mapped and explored with a variety of additional features (e.g., from CATH, COSMIC, PredictProtein, SNAP2, and UniProt).

Conclusions

We believe our tool is the first to provide a simplified way of (1) mapping any variant onto essentially any protein structure, and (2) exploring mapped structures using XR. Thus, our tool may enable many life scientists to benefit from the wealth of detail previously buried in structural data.

P04.01

Ghost Proteome Revealed Involved in Functional Regulator of Glioma Using Crosslink Mass Spectrometry

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Introduction: Ghost proteins are issued from alternative Open Reading Frames (AltORFs) and are missing a genome annotation. This Ghost proteome was neglected, and one major issue is to identify the implication of the alternative proteins (AltProts) in the biological processes. We aimed to identify the protein-protein interactions (PPIs) by large scale analysis using crosslink mass spectrometry (XL-MS), of the RefProt and AltProt in the context of cancer cell. Particularly in the cellular reprogramming appearing in glioblastoma cells under stimulation with Forskolin, inducing an astrocyte differentiation.
Method: NCH82 human glioma cells which were stimulated by the protein kinase A activator Forskolin to induce cell differentiation, a LFQ analysis of the RefProt is performed, XL-MS is realized using the DSSO, to allow us to simplify the sample. Data are analyzed using Proteome Discoverer 2.3 with the node XLinkX, and network drawing and interpreted from Cytoscape. Some of AltProt-RefProt interaction, were observing in cells after co-localization of the proteins expressed in cell with FLAG and GFP.
Results: The data shows us an important change in the protein level in the cells, after stimulation with Forskolin. LFQ performed on the RefProt suggests an impact on the cell reprogramming, by identification of several actors, like TGFβ1, DIDO1, MAP4K4, HOOK3. Other pathway were describe to be involved, like the over-expression of the mitochondrial tRNA processing or the under-expression of the cytosolic tRNA aminoacylation.
Conclusion: Crosslink analysis of RefProt and complemented with AltProt database, partners identification are enriched with the STRING annotation and interrogated with ClueGO. This allows us to give a RefProt annotated partner to the unknown AltProt and correlate the Gene Ontology annotation of the partner to the AltProt in the aim to access at a first idea of the role in the cell.

P04.02

Proteomic Investigation of Stress-Induced Neurological Changes in Brain Regions of an Alzheimer's Disease Transgenic Mouse Model

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Introduction: Alzheimer's Disease (AD) is a neurodegenerative disease and the most common cause of dementia worldwide. Despite decades of investigations, the etiology of AD is not fully elucidated, however emerging evidence suggest that chronic environmental and/or psychological stress is involved in the process and contribute to the risk of developing AD. Thus, understanding the impact of stress on the AD-brain might further our understanding of pathological mechanisms involved in AD development. We present the proteomics investigation of the effects of a Chronic Stress model on the proteome of APPPS1 transgenic and wildtype (WT) mice.

Methods: APPPS1 and WT mice were subjected to Chronic Stress for 4 weeks including diurnal disruption (light/dark schedule: 10L/10D), food deprivation, cage tilt, water deprivation and confinement. Control mice of both genotypes were kept under standard conditions. The chronic stress period was followed up by 3 weeks with only diurnal disruption, after which mice were euthanized and cortex, hippocampus, middle brain and brain stem were dissected out and used for label-free quantitative DIA based LC-MS/MS analysis. The right hemispheres were coronally cut on a cryostat and used for imaging mass spectrometry of metabolites accomplished by using MALDI2 based MSI of whole brain slices.

Results: In all investigated brain regions, quantitative proteome DIA profiling identified significantly up- and downregulated proteins in both APPPS1 and WT mice with chronic stress treatment compared to the respective controls groups. Protein interaction network mapping was used to annotate these proteins to pathways of interest, including the immune system, mitochondrial function (including TCA cycle and oxidative phosphorylation), cell death and AD.

Conclusion: Chronic stress changes the proteome in the specific brain regions in both APPPS1 and WT, with the largest impact on the hippocampus and mitochondrial function in this tissue. MSI using MALDI2 significantly improved the detection of affected metabolites and lipoproteins.

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P04.03

Deep Proteomic Profiling of Alzheimer's Disease CSF for Unbiased Biomarker Discovery and Subject Stratification

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INTRODUCTION
Cerebrospinal fluid (CSF) is established as a key matrix that enables interrogation of biological processes within the central nervous system. CSF biomarkers may support development of new therapies through patient stratification, determining prognosis or disease aggressiveness, and response monitoring. However, the need for better biomarkers and biological understanding is evidenced by the lack of success of disease modifying drugs in late-stage clinical trials. Here, we seek to address this unmet need by applying an optimized workflow to deeply characterize the proteomes of CSF from subjects with Alzheimers' Disease (AD).

METHODS
CSF samples were obtained from subjects with late onset AD (n = 16) and age-matched normal controls (CO; n = 8). The samples were processed using in solution digestion and subsequently analyzed using a Thermo Scientific Orbitrap Exploris 480 equipped with a FAIMS Pro device. Data processing and analysis were performed using Biognosys' SpectroMine and Spectronaut software.

RESULTS
Across all samples we identified and quantified 3,521 unique proteins with high quantitative accuracy from 51,075 peptides in single shot acquisitions. the depth and breadth of protein quantification covers numerous pathological mechanisms including AB and Tau pathology, synaptic dysfunction, iron toxicity and inflammation. Next, we interrogated the obtained proteome dataset using a combination of peptide-centric analyses focusing specifically on semi-tryptic peptide species. Among all identified peptides, 38% exhibited semi-tryptic digestion profiles. Importantly, in AD patients significantly ($p = 0.038$) lower levels of semi-tryptic peptide-species were identified compared to age-matched control individuals, indicating that certain peptide species derived from post translational processes could be de-regulated in Alzheimer's Disease.

CONCLUSIONS
The presented workflow enables simultaneous quantitative characterization of 3,500 CSF proteins, covering >90% of known markers and is scalable to monitor 100s - 1000s of samples. Peptide-centric analysis provides an additional layer of information regarding post-translational processing, truncation and proteolytic events.

P04.04

Dynamics of Huntingtin Protein Interactions in the Striatum Identifies Candidate Modifiers of Huntington's Disease

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Introduction: Huntington's disease (HD) is an autosomal dominant fatal disease that affects about one out of every 10,000 individuals in the US. HD is a monogenic neurodegenerative disorder with one causative gene, huntingtin (HTT). Yet, the pathobiology is multifactorial, suggesting that cellular factors influence disease progression. Towards this goal, we defined HTT protein-protein interactions (PPIs) perturbed by the mutant protein with expanded polyglutamine (mHTT) that may be proximal disease modifiers and therapeutic targets.

Methods: Dissected striatum of 2- and 10-month-old mice expressing 3XFLAG-Htt with a knock of human exon 1 containing either normal (20Q) or expanded (140Q) polyglutamine tracts (1) were used for label-free and isotope-labeled affinity purification-mass spectrometry experiments. Label-free and isotope labeled AP-MS determined the specificity and relative stability of the interacting protein as previously described (2-3), except here adapted for tissues. Polyglutamine-regulated PPIs were validated in a human HD cell model using dual-readout bioluminescence-based two-hybrid assays (4).

Results: Using metabolically labeled tissues and immunoaffinity purification-mass spectrometry, we establish the poly-glutamine and disease stage-dependent modulation of HTT PPI abundances and relative stability in the mouse striatum, a brain region with selective HD vulnerability. Validated PPIs include modulators of vesicular transport (SNAREs) and synaptic transmission (glutamatergic receptors). PPIs were modulated prior to disease manifestation, notably regulators of actin polymerization, including the Arp2/3 complex, which displayed increased relative stability.

Conclusions: Using complementary AP-MS approaches and in-cell two hybrid assays, we discover leading candidates for proximal disease modifiers of Huntington's disease. Our findings provide a resource for future studies of HD cellular pathobiology.

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P04.05

Brain Glycoproteomic Network Alterations in Alzheimer’s Disease

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Introduction: Alzheimer's disease (AD) is a devastating dementia. Protein glycosylation plays critical roles in controlling brain function, but our knowledge of human brain glycoproteome is limited, and disease-associated glycoproteome changes in AD brain remain mostly undefined. To address the knowledge gap, we established a systems biology approach that integrates quantitative proteomics, site-specific glycoproteomics, and glycopeptide/glycoprotein co-regulation network analysis and applied this approach to identify protein glycosylation aberrations and glycoproteomic network alterations in AD brain.
Methods: Large-scale, site-specific, quantitative glycoproteomics analyses of human AD and control brain tissue samples were performed using a mass spectrometry-based glycoproteomics approach. The glycoproteome data were integrated with the proteome profiling data from the same brain samples to identify altered glycopeptides, glycoproteins, and glycosylation site occupancy in AD brain. Proteomics- and glycoproteomics-driven network analyses were performed to identify protein co-expression network and glycopeptide/glycoprotein co-regulation network alterations in AD brain.
Results: Our integrative glycoproteomics and proteomics analyses uncovered disease signatures of altered glycopeptides, glycoproteins, and glycosylation site occupancy in AD brain. We found that human brain glycoproteome is organized into a glyco-network of 13 modules of co-regulated glycopeptides/glycoproteins. The glycoproteome network has no significant overlap with the proteome network from the same brain samples, indicating that glycopeptide/glycoprotein co-regulation and protein co-expression are controlled by different mechanisms. Module-trait association analyses identified 6 glyco-network modules that are associated with AD clinical phenotype, amyloid-β and/or tau pathology. Our study revealed a number of dysregulated glycosylation-affected processes in AD brain, including extracellular matrix dysfunction, neuroinflammation, synaptic dysfunction, cell adhesion alteration, lysosomal dysfunction, endocytic trafficking dysregulation, endoplasmic reticulum dysfunction, and cell signaling dysregulation.
Conclusions: This work provides a systems-level view of human brain glycoproteome and reveals previously unknown, glycosylation aberrations and glycoproteomic network alterations in AD brain. Our findings provide new molecular and systems-level insights for understanding and treating AD.

P04.06

Omics Insights into Gender Differences in Alzheimer Disease Subjects

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Introduction: Neuropathology of Alzheimer's disease (AD) has been well defined, but the underlying causes of the disease remain debatable. Recently, gender and glutamatergic neurotransmission are emerging as crucial drivers of development and progression of AD dementia. We evaluated by an integrated multi-omics approach the alterations observed in hippocampal regions of AD patients compared to healthy individuals taking into consideration the gender contribution.
Methods: A principal component analysis was carried out first by grouping and averaging quantitative data in the sample groups: CTR and AD, and then considering males (M) and females (F) as separate subgroups of each. The analysis was conducted considering proteins differentially expressed if they were present only in one condition or showed significant T-test difference.
Results: There is a significant difference between male and female in all the omics data sets either in the neurodegenerative disease and in the control. Bioinformatics analysis highlight major differences in energetic metabolism (TCA cycle, glycolysis or gluconeogenesis, amino acid, pyruvate, glyoxylate and dicarboxylate metabolism), neuronal system and post-synapse organization, cytoskeleton organization, oxidative stress response and inflammation. The analysis suggests that the neurodegenerative disease changes and for some respects almost reverses the proteomic and metabolomic profile peculiar to males and females.
Given the association recently discovered between AD progression and D-serine level (D-serine is the main coagonist of the synaptic NMDA receptors), we focused also on gene and protein expression and metabolite levels of the serine biosynthesis pathway. Our data suggest that serine metabolism, connected to NMDAR receptor activation, reveals genders differences in hippocampal brain tissues.
Conclusion: The results are of main relevance for future clinical interventions that can reduce dementia risk in both male and female patients.
This project was founded by "PRIN2017:2017H4J3AS Dissecting serine metabolism in the brain".

P04.07

Identification of Proteins Altered in Alzheimer's Disease by Mass Spectrometry That Could Be Key for the Understanding of the Disease

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Introduction and objective

Alzheimer's disease (AD) is a progressive, chronic and neurodegenerative disease, which is currently the most common form of dementia worldwide. The mechanisms underlying the disease are not well known. Thus, the study of proteins involved in its pathogenesis would allow getting further insights into the disease and identifying new potential markers for early AD diagnosis. We here aimed to analyze protein dysregulation in AD tissue by quantitative proteomics to identify proteins that could play a major role in the disease.

Methods

TMT (Tandem Mass Tags) 10-Plex-based quantitative proteomics experiments were performed using frozen tissue samples from the left prefrontal cortex of AD patients at Braak stages IV-VI, and healthy individuals and patients with other dementias (vascular and frontotemporal dementia) as controls. LC-MS/MS analyses were performed on a Q-Exactive, and data analysis was performed with MaxQuant and Perseus to identify differentially expressed proteins in AD.

Results and discussion

In total, 3281 proteins were identified and quantified with MaxQuant. After data analysis, we observed 31 and 250 proteins upregulated and downregulated, respectively, in AD patients in comparisons to controls, with a fold change ≥ 1.5 or ≤ 0.67. After bioinformatics analysis, we selected 10 proteins dysregulated in AD to study their role in the pathogenesis. Furthermore, their dysregulation in the disease was verified by orthogonal techniques (qPCR, WB, IHC and ELISA) using tissue and serum samples of AD patients, patients with other dementias and healthy individuals.

Conclusion

TMT-based quantitative proteomic experiments allowed the identification of proteins altered in AD previously and non-previously related to the disease. The dysregulation of these proteins at mRNA and protein level was confirmed in AD patients, highlighting a major role of these proteins in the development of the disease and as new biomarkers and/or potential therapeutic targets of intervention.

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P04.08

Proteomic Analysis of Three Brain Regions Isolated from Patients with Mesial Temporal Lobe Epilepsy Reveals Molecular Alterations beyond the Hippocampus

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Introduction: Epilepsy is a chronic neurological disorder affecting 2% of the world population. It presents a wide variety of clinic manifestations, etiologies, severity, and prognosis. Nevertheless, the occurrence of epileptic seizures caused by abnormal neuronal discharges is the characteristic feature of all types of epilepsy. Mesial temporal lobe epilepsy (MTLE) accounts for around 40% of adult patients with epilepsy, many of whom do not respond well to clinical treatment. In these patients, the main lesion is thought to be mainly restricted to the medial temporal structures, mainly the hippocampus. Epilepsy surgery is a well-recognized treatment option for patients with MTLE and refractory seizures, and the tissue resected can be subsequently studied. In this work, we aimed to perform large-scale proteomics in different brain structures from patients with MTLE in different stages of the disease.
Methods: We analyzed hippocampus, amygdala, and temporal neocortex (N=10 for each structure) isolated from the same patients using S-Trap columns, TMT10-plex, high pH reverse phase fractionation, and a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer. For the analysis, we used Proteome Discoverer 2.4 and R software.
Results: We identified a total of 7727 proteins, and the major altered biological processes were: cellular processes, metabolic processes, biological regulation, and localization. The main enriched pathways were: integrin signaling pathways, Wnt signaling pathway, inflammation mediated by chemokines, gonadotropin-release hormone receptor, and CCKR signaling pathway. Furthermore, the proteomic abnormalities observed changed according to disease duration.
Conclusion: Our data brings new information regarding the array of molecular abnormalities occurring in MTLE. It also suggests that there is a progression of the lesion identified in patients with MTLE. Finally, we have evidence that molecular abnormalities are occurring beyond the hippocampus in these patients.

P04.09

Differential Proteomic Analysis of Astrocytes and Astrocytes-Derived Extracellular Vesicles from Control and Rai Knockout Mice: Insights into Neuroprotective Mechanisms

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Introduction: Reactive astrocytes are a hallmark of neurodegenerative diseases including multiple sclerosis (MS) and they may assume alternative phenotypes depending on a combination of environmental signals and intrinsic features; however, we still lack a full understanding of their role in the disease. Previous results have shown in the experimental autoimmune encephalomyelitis (EAE) mouse model that deficiency of the molecular adaptor Rai reduces disease severity and demyelination. Therefore, we investigated the impact of Rai expression on astrocyte function and astrocytes-released extracellular vesicles (EVs) both under basal conditions and in response to IL-17 treatment via a proteomic approach.
Methods: A differential proteomic analysis of astrocytes and astrocytes-derived EVs was performed. Enrichment and network analyses were performed by MetaCore and validations of data were carried out by qRT-PCR and WB.
Results: A dysregulation of various proteins, to which Rai contributes, was evidenced in astrocytes and astrocyte-derived EVs and they are mainly involved in the regulation of oligodendrocyte differentiation and myelination, nitrogen metabolism, proteasome-ubiquitin pathway and oxidative stress response. Indeed, cell viability assay data highlighted the ability of Rai deficient astrocytes to survive extracellular ROS, when compared with controls. Pathway analysis comparison of differential proteins between astrocytes and EVs highlighted the shared involvement of the HIF-1 pathway and cellular energetic metabolism as the most statistically relevant molecular pathways, associated with ENOA and HSP70. Indeed, a downregulation of HIF-1α transcripts was observed in Rai knockout mice, independently on the IL-17 stimulation, as well as of ENOA protein, together with an upregulation of HSP70 in Rai knockout IL-17 stimulated mice.
Conclusions: Our findings highlight Rai as a novel participant in the yet largely unknown signalling pathways driving astrocytes response to pro-inflammatory signals and that its deficiency contributes to the establishment and mediation of neuroprotective responses.

P04.10

Assessment of IsoAsp7 Amyloid-beta Peptides as a Perspective Diagnostic Target of AD Progression by Proteomic MS Based Approaches

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Introduction
Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of insoluble aggregates of Aβ peptides in the brain tissues. Isomerization of Asp7 in Aβ is known to provide neurotoxicity trigger cerebral amyloidosis in vivo and indicates AD progression. Animal models are critical for understanding disease pathogenesis and also serve as valuable tools for preclinical testing. The purpose of this study is to quantify the ratio of normal and isoAsp7-Aβ in brain tissues of FADx5 mice of different ages, as well as in human AD brain samples by mass spectrometry-based proteomics tools.
Methods
To isolate Aβ, the brain tissue homogenate was subjected to FA extraction followed by SPE. For MS analysis, a truncated Aβ(1-16) fragment was obtained by hydrolysis. To quantify the proportion of isoAsp7- Aβ, the intensities of isoform specific characteristic peaks in the MS/MS spectra of Aβ(1-16) from brain samples were measured. The samples were analyzed using a MALDI-TOF/TOF UltrafleXtreme mass spectrometer. A complementary study was carried out using the separation of isoforms by ion mobility and liquid chromatography on a TIMS-TOF Pro instrument.
Results
The analysis of Aβ-rich fractions obtained from the brains of transgenic mice aged from 4 to 16 months was carried out. According to the results, a trend towards the accumulation of isoAsp7- Aβ with increasing age was observed (up to 20%). The ratio of iso/norm Aβ in human AD brains was much higher with isoAsp7 present in 70-90% of Aβ. The results obtained using a MALDI-TOF/TOF instrument are consistent with the TIMS-TOF Pro measurements.
Conclusion
The data obtained for mice of different ages confirm the hypothesis about the relationship between the accumulation of isoAsp7-Aβ and the progression of AD-like pathology. However, the result for mice is significantly different from that for humans, presumably due to the limited lifespan of the animals.

P04.11

Proteomic Profiling Provides New Insights into the Role of Neuromelanin Granules in Neurodegenerative Processes

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Introduction
Neuromelanin is a black-brownish pigment, present in so-called neuromelanin granules (NMG) in dopaminergic neurons of the human substantia nigra pars compacta. Besides the pigment neuromelanin, NMG also contain a variety of proteins, lipids and metals. Their clinical relevance is based on the observation that especially neuromelanin-containing dopaminergic neurons are lost during neurodegenerative diseases like Parkinson's disease (PD) and dementia with Lewy bodies (DLB). However, the role of NMG in neurodegenerative processes and the mechanisms of their formation are mainly elusive. We therefore aimed to gain a deeper insight into the NMG composition and ultimately functionality by comparing the proteomic profile of NMG and surrounding tissue (SN) of healthy controls and DLB patients.

Methods
In order to analyze the NMG proteome, NMG and SN tissue were isolated from post-mortem brain slices of healthy controls and DLB patients via laser capture microdissection. Subsequently, the samples were processed for mass spectrometry-based analysis using LC-MS/MS. Mass spectrometry data was analyzed with MaxQuant software using a label-free quantitative approach with subsequent statistical evaluation. Significantly differential proteins were selected for validation by parallel reaction monitoring (PRM) experiments, determining their abundance in the different tissues and health states.

Results
Proteomic profiling of NMG led to the identification of 2000 proteins. Quantitative comparison with SN tissue proteome allowed the identification of NMG-associated proteins. Several proteins with significantly higher abundance in NMG were found to be associated with translation and RNA metabolism. Additionally, several RNA-binding proteins known to be associated with neurodegenerative diseases were of higher abundance in NMG as well. Furthermore, the quantitative comparison of NMG proteome and SN proteome in health and disease revealed various proteins to be differentially abundant.

Conclusions
In general, our study indicates a yet undescribed origin of neuromelanin granules and gives new insights into their role in neurodegenerative diseases.

P04.12

Mitochondrial, Cell Cycle Control and Neuritogenesis Alterations in an IPSC-Based Neurodevelopmental Model for Schizophrenia

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Introduction: Schizophrenia is a severe psychiatric disorder of neurodevelopmental origin that affects around 1% of the world's population. Proteomic studies and other approaches have provided evidence of compromised cellular processes in the disorder, including mitochondrial function. Most of the studies so far have been conducted on postmortem brain tissue from patients, and therefore do not allow the evaluation of the neurodevelopmental aspect of the disorder. Methods: To circumvent that, we studied the mitochondrial and nuclear proteomes of neural stem cells (NSCs) and neurons derived from induced pluripotent stem cells (iPSCs) from schizophrenia patients versus healthy controls to assess possible alterations related to energy metabolism and mitochondrial function during neurodevelopment in the disorder. Results: Our results revealed differentially expressed proteins in pathways related to mitochondrial function, cell cycle control, DNA repair and neuritogenesis and their possible implication in key process of neurodevelopment, such as neuronal differentiation and axonal guidance signaling. Moreover, functional analysis of NSCs revealed alterations in mitochondrial oxygen consumption in schizophrenia-derived cells and a tendency of higher levels of intracellular reactive oxygen species (ROS). Conclusions: Hence, this study shows evidences that alterations in important cellular processes are present during neurodevelopment and could be involved with the establishment of schizophrenia, as well as the phenotypic traits observed in adult patients.

P05.02

A Survey of the Cancer Conformational Landscape Establishes Novel Anti-Cancer Drug Targets

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Introduction
The prevailing paradigm in cancer research states that somatic DNA mutations derail genes [1,2] and alter protein sequences to cause cancer and trigger adaptive cellular responses that contribute to tumorigenesis [3,4]. However, it is currently impossible to prioritize a first-line therapy based solely on the somatic mutation pattern of evolving tumors, in part because the impact of somatic mutations on protein folding and protein-protein interactions during malignant transformation remains unknown.
Methods
To detect relative changes in the structural proteome we developed covalent protein painting (CPP), a novel, high throughput mass spectrometric method to quantitatively measure alterations in protein structure and interactions [5]. Here, we used CPP to survey all 60 cell lines of the anti-cancer cell line panel (NCI60) for lysine sites that changed in accessibility for chemical modification.
Results
We quantified 8,025 lysine sites in ≥3,000 protein groups and found that >5 structural alterations differentiate any cancer cell line from the other 59. Structural aberrations in 98 proteins correlated with the presence of 90 commonly mutated proteins, suggesting a more complex association of genotype to effector protein conformation. For example, de novo mutation of H1047R in phosphatidyl-inositol kinase 3 p110alpha (PIK3CA) in MCF10A cells [6] altered the accessibility to 24 out of 2,798 (0.8 %) lysine sites, but these 24 sites did not include the 7 sites commonly found to be altered in cell lines that harbor oncogenic PIK3CA [7]. Further, we identified 49 conformational alterations in the cancer conformational landscape that correlated with the growth inhibition profiles of 300 out of 50,000 small molecule drug candidates [8]. We found that 3D alterations in heat shock proteins of the proteostasis network are key predictors of anti-cancer drug efficacy.
Conclusions
In summary, cancer-associated structural alterations may be biomarkers for malignant transformation and may provide leads for anti-cancer drug development.

P05.03

Proteomics and Large-Scale, Comparative Cross-Linking Mass Spectrometry Reveal Novel Roles for Ribosome Histidine Methylation

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Introduction
Recent studies have uncovered evidence for hundreds of histidine methylated proteins, with histidine methyltransferases themselves increasingly implicated in human disease. Hpm1 (orthologous to human METTL18) represents the only histidine methyltransferase in Saccharomyces cerevisiae. It mono-methylates H243 of the ribosomal protein Rpl3. Interestingly, the hpm1 deletion strain is highly pleiotropic, with many extra-ribosomal phenotypes including an improved growth rate in many alternative carbon sources. Through a combination of quantitative proteomics and cross-linking mass spectrometry, here we aimed to understand how methylation of an amino acid in one ribosomal protein could result in such diverse phenotypes.

Methods
Wild-type and hpm1 knockout yeast were cultured using forward/reverse SILAC. For proteomics analyses, digests were fractionated by SCX and analysed with data-dependentent LC-MS/MS analysis, with downstream analysis using MaxQuant and Proteus. For cross-linking, intact spheroplasts were crosslinked with the biotin-containing and MS-cleavable crosslinker PIR. Peptides were fractionated by SCX, enriched with avidin and analysed using a stepped-HCD MS2 method. Crosslinks were identified using the Mango-Comet-XlinkProphet pipeline, and quantified using MethylQuant. Matched proteome analyses were performed to control for protein abundance changes.

Results
Proteomics revealed 30 differentially-abundant proteins, most with clear links to the coordination of sugar metabolism. We successfully adapted the emerging technique of quantitative large-scale cross-linking mass spectrometry to study the in vivo dynamics of protein interactions and structures in budding yeast for the first time. By reproducibly monitoring over 350 unique-residue-pairs, we were able to detect changes to membrane protein structure, chromatin compaction, and mitochondrial protein-protein interactions, independently of changes in protein abundance themselves.

Conclusions
Together these studies contextualise the pleiotropic Δhpm1 phenotype. They provide new insights into histidine and ribosomal methylation, important for human and other eukaryotic systems where Hpm1 is conserved. They illustrate how cross-linking mass spectrometry can generate unbiased, mechanistic insights into complex cellular processes.

P05.04

Assessing Therapeutic Diet-Induced Succinylome Remodeling in Injured Kidney and Liver using Library-Free Data-Independent Acquisition

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Introduction
Ischemia/reperfusion-induced acute kidney injury (AKI) causes kidney dysfunction and oxidative stress. We have previously shown that Sirtuin 5 (SIRT5, a de-succinylase) knockout (KO) in mouse kidney increases succinylation levels, and promotes the fatty acid oxidation switch from mitochondria to peroxisomes, reducing oxidative stress and protecting against AKI. Here, we investigate the beneficial effects of succinylome remodeling in mouse injured kidney and liver in response to Sirt5-KO and/or nutritional supplement NS-1 regimen.

Methods
To induce AKI, one kidney was subjected to ischemia, while the contralateral one was used as control. Moreover, mice were fed with NS-1 or normal diet. For the liver study, Sirt5-KO and WT mice were also subjected to NS-1 or normal diet. After succinyl peptide enrichment using PTMScan immunoaffinity beads (CST), samples were analyzed by data-independent acquisition (DIA) on an Orbitrap Eclipse Tribrid platform, and data were processed without pre-existing spectral libraries using directDIA (Biognosys).

Results
To explore the dynamic changes of lysine succinylation, we optimized a powerful emerging strategy coupling PTM enrichment and refined library-free DIA workflows. In the kidney ischemia model, 3,666 succinylated sites were quantified. Diet induced hypersuccinylation of 1,085 sites in injured kidney, while the proteome was minimally affected (26 proteins). Upregulated succinylation sites were related to lipid and fatty acid oxidation, and peroxisomal metabolic pathways.
Moreover, succinylation profiling in liver from Sirt5-KO and WT mice revealed a large diet-induced remodeling, with 704 upregulated sites, mostly from peroxisomal proteins, and 265 downregulated sites, mostly from mitochondrial proteins, highlighting an organelle-specific regulation.

Conclusions
The efficient DIA proteomics workflow enabled gaining insights into liver and kidney responses upon diet, which improved injury phenotypes and metabolic activities. Altogether, this demonstrates the promising translational and therapeutic applications of NS-1 to rescue human injury phenotypes, and provide alternative energy sources for patients with genetic disorders affecting mitochondrial metabolisms.

P05.05

Interactome Analyses and HDX-MS Reveal Profound Proteasome Structural and Functional Rearrangements throughout Mammalian Spermatogenesis

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Introduction: The proteasome is a complex molecular machinery whose main role is to degrade proteins. Catalytic subunits can be replaced by tissue-specific subunits, giving rise to proteasome subtypes performing particular functions (constitutive proteasome c20S, immunoproteasome i20S). The proteolytic activity is regulated by different protein complexes, such as the 19S, PA28s, PA200. The subunit called α4s is specific for gamete cells, where it replaces the standard α4, creating the spermatoproteasome (s20S) which has recently been shown to be indispensable in spermatogenesis. Knowing which proteins s20S interacts with and deciphering the nature of these interactions could help understand why is α4s so crucial.
Methods: We approached this question by: 1- exploring the dynamics of proteasome interactome using affinity purification strategies and shotgun proteomics and 2- looking at structural differences between the c20S and s20S complexes using a structural mass spectrometry technique called Hydrogen-Deuterium eXchange (HDX).
Results: After analyzing the germ cells at different stages of development, we observed that the s20S becomes highly activated as germ cells enter meiosis, mainly through a particularly extensive 19S activation and, to a lesser extent, PA200 binding. Additionally, the proteasome population shifts from predominantly c20S to predominantly s20S during differentiation, presumably due to the shift from α4 to α4s expression. We demonstrated that s20S, but not c20S, interacts with components of the meiotic synaptonemal complex, where it may localize via association with the PI31 adaptor protein. In vitro, s20S preferentially bind to 19S, and displayed higher trypsin- and chymotrypsin-like activities, both with and without PA200 activation. Moreover, using MS methods to monitor protein dynamics, we identified significant differences in domain flexibility between α4 and α4s.
Conclusion: We propose that these differences induced by α4s incorporation result in significant changes in the way the s20S interacts with its partners, and dictate its role in germ cell differentiation.

P05.06

A Structural Analysis of Heated Ovalbumin by Crosslink Proteomics

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Introduction: Egg allergy affects 1.3-1.6% of children and usually develops in the first year of life (1). A major allergen implicated in egg allergy is ovalbumin (OVA, Gal d 2), the main protein of egg white. Studies have pointed out that between 50 and 85 % of egg-allergic patients can tolerate cooked or extensively heated eggs, suggesting a link between heat-induced structural modifications of egg allergens and allergenicity (2,3). Although the heat-induced structural modifications of OVA modulate its allergenicity, little molecular data are available to explain this change.

Methods: Heat-induced crosslinks and glycation sites in OVA following aggregation and glycation by different processes were assessed by an innovative mass-spectrometry approach using dedicated software (pLink, Merox & PTMProphet)

Results: We successfully identified and mapped aggregation and glycation sites of heated and glycated OVA. Our results show that aggregation is mainly driven by disulfide bond formation at close proximity of known human linear IgE epitopes. Furthermore, our results suggest that the sites of glycation in OVA are dependent on the type of process used. Several of the identified aggregation and glycation sites are in close proximity to known OVA epitopes.

Conclusions: Structural modifications of OVA following thermal treatment appear to masks specific OVA epitopes, thereby possibly contributing to the reduced allergenicity of heated OVA.

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P05.07

N-Glycoproteome from a Cancer Cell Line and Its Non-tumorigenic Cell Line Combining Fbs1-GYR N-Glycopeptide Enrichment and Trapped-Ion-Mobility-Quadrupole-Time-of-Flight

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Introduction
N-glycosylation is implicated in the development and progression of many types of cancer. Analysis of N-glycopeptides is an analytical challenge for LC-MS/MS with respect to electrospray ionization, chromatographic separation and structural elucidation. In this work, we have combined Fbs1-GYR N-glycopeptide enrichment technology with parallel accumulation serial fragmentation (PASEF) on a trapped ion mobility spectrometry (TIMS) - quadrupole time-of-flight (QTOF) mass spectrometer to study the comprehensive glycopeptide profiles in HCT116 cancer cells and their non-tumorigenic DNMT1/3b double knockout cells (DKO1).

Methods
N-glycopeptides from HCT116 and DKO1 cells were enriched by Fbs1-GYR. The enriched samples were loaded onto a nanoELUTE coupled to a timsTOF Pro (Bruker Daltonics) using a reverse-phase C18 IonOpticks Aurora nano column run over a 45-minute or 90-minute gradient. Data was searched in Byos (Protein Metrics Inc.) against the Uniprot human database and a human glycan database containing 132 N-glycan structures. The number of peptide-spectrum matches (PSMs) were used for glycosylation quantification/comparison.

Results
Using the global proteome we could identify around 29,000 and 31,000 PSMs for HCT116 and DKO1 cells, respectively. However, just around 0.4% of the PSMs came from N-glycopeptides. By applying Fbs1-GYR enrichment technology, the number of PSMs increased to 60,000 PSMs for both cell lines with PSM values for N-glycopeptides corresponding close to 45%. Thus, Fbs1-GYR enabled more than 100-fold enrichment of N-glycopeptides. Moreover, over 1,000 glycoproteins and 2,300 N-glycosites could be identified. Label free quantitative analysis for differential expression was performed and compared using a T-test, identifying hundreds of potential cancer biomarkers for this specific cell line. Among several differentiated glycans compositions for these two different cell lines, mannose-6-phosphate (M6P) modification showed to be up-regulated (3.7-fold) in the cancer line. Interestingly, glycopeptides showed a mobility offset mass aligned (MOMA; i.e. same m/z and retention time, distinct mobility) suggesting different glycoforms for a given glycopeptide.

P05.08

Proteomics of A-to-I Rna Editing in Mouse and Human

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Introduction

Editing of A-to-I in RNA is a type of post-transcriptional modification done by specific deaminases acting on RNA (ADAR). In the case of coding regions editing it leads to the recoding of proteins and these events can be tracked on the level of proteome. In our work, we implemented a proteogenomic approach to study protein products of RNA editing of this kind.

Methods

Briefly, the proteogenomic approach consists of database construction, proteomic search, and result filtering and validation. We have taken the available transcriptomic data for mouse and human editomes and translated them to proteomic databases. After that, proteomic searches were performed using X!Tandem search engines. The results were filtered according to group-specific principle to 1% FDR using the target-decoy approach.

Results

For murine RNA editing proteomic search, we have chosen several datasets representing deep proteomes of the mouse brain regions and neuronal cell cultures. We have found 20 significant sites of editing. Some of the sites demonstrated differential distribution between brain cell types.

For the human RNA editing study, 40 available datasets were selected. Unlike the model organism, the human study requires the account of genetic polymorphisms that make the data analysis more complicated. A total of 37 edited sites were found. Most of the sites were found in the brain and brain vessel tissues. Of them, 10 were homologous with the murine sites.

Conclusions

The proteogenomic approach can be used to trace coding RNA editing events on the proteomic level. Only very limited portion of the sites predicted by transcriptomics is found in the proteome. There were 8 sites shared between murine and human brain proteomes. The editing of these proteins is known to play a crucial role in the living of an organism.

P05.09

Mapping the Functional Proteome Landscape of Escherichia Coli with Thermal Proteome Profiling

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Introduction
Systematic mapping of protein function and interactions has been enabled by high-throughput reverse genetics. However, these approaches integrate all the molecular changes in morphological readouts, which hinder mechanistic understanding of the effects of a perturbation. Here, we combined a reverse genetics approach with thermal proteome profiling (TPP) to measure proteome-wide abundance and thermal stability of genetic perturbations in Escherichia coli.

Methods
We performed TPP on 121 single gene deletion mutants of E. coli, perturbing virtually all key cellular processes. Samples were multiplexed, fractionated, and analyzed on Orbitrap instruments. We validated our findings by using metabolomics, and computational and genetic tools.

Results
We measured abundance and thermal stability of nearly 1,800 proteins across the different perturbations. This revealed that essential proteins are rarely regulated in their abundance, but commonly change in their thermal stability and thus their activity. We found that functionally associated proteins have coordinated abundance and thermal stability changes across mutants, which are a result of their co-regulation and physical interactions (with metabolites, co-factors or other proteins). This allowed us to suggest the function of uncharacterized proteins in a guilt-by-association manner. We further observed that enzyme thermal stability was correlated with metabolite levels, as measured by targeted metabolomics. Finally, our proteome measurements were able to explain the molecular mechanisms of growth phenotypes in different chemical and environmental perturbations.

Conclusions
Combining the benefits of systematic reverse genetics approaches with multi-parametric proteome readouts allows mapping the proteomic landscape to an unprecedented scale, by determining protein states and interactions directly in situ. The data represents a rich resource for inferring new protein functions and interactions, and the approach is readily expandable to other organisms.

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P05.11

HaDeX 2.0: Web-Server and R Package for the Hydrogen-Deuterium Exchange Mass Spectrometry Experiments Data

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Introduction: Hydrogen-deuterium mass spectrometry (HDX-MS) is an analytical tool for monitoring the dynamics and interactions of proteins. In contrast to crystallography-based methods, HDX-MS allows a unique insight into the dynamics of the protein structure. Such data is very complex and requires a dedicated solution, like our tool HaDeX (1). However, our growing user base motivated us to greatly improve our software by adding new functionalities and polishing the existing ones leading to the HaDeX 2.0.
Methods: HaDeX 2.0 is a versatile software for processing, analyzing, and visualizing output data from existing tools used in HDX-MS experiments as DynamX, HDeXaminer, or Mass Spec Studio. HaDeX provides a complete analytic workflow, with precise uncertainty calculations (2) and report generation to ensure reproducibility as recommended in the community guidelines (3). HaDeX is freely available as a unique combination of web-server (https://hadex.mslab-ibb.pl/), standalone application, and an open-source R-package.
Results: The application is significantly extended compared to its prior version. HaDeX 2.0 contains all forms of visualizations employed by the HDX-MS community, e.g., chiclet, butterfly, and volcano plots. All of the figures are of publication quality, featuring ISO-based uncertainty of the measurement. Moreover, HaDeX fully supports differential analysis in the form of charts and a novel semi-parametric statistical test. The replicate analysis module allows an in-depth inspection of the data, further increasing the quality control capabilities of HaDeX.
Conclusions: As HDX-MS is getting more recognition, there is a growing need for standardized analysis and data processing following the community recommendations. Our answer to this issue is HaDeX 2.0, an open-source software ready to tackle all challenges associated with the analysis of the HDX-MS data.

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P05.12

Prolonged Exposure to Traffic-Related Particulate Matter and Gaseous Pollutants Implicate Distinct Molecular Mechanisms of Lung Injury in Rats

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Background: Exposure to particulate matter (PM) pollution exerts direct effects on respiratory organs; however, molecular alterations underlying PM-induced pulmonary injury remain unclear. Methods: In this study, we investigated the effect of PM on the lung tissues of Sprague-Dawley rats with whole-body exposure to traffic-related PM1 (<1 μm in aerodynamic diameter) pollutants and compared it with that in rats exposed to high-efficiency particulate air–filtered gaseous pollutants and clean air controls for 3 and 6 months. Lung function and histological examinations were performed along with quantitative proteomics analysis and functional validation.
Results: Rats in the 6-month PM1-exposed group exhibited a significant decline in lung function, as determined by decreased forced expiratory flow and forced expiratory volume; however, histological analysis revealed earlier lung damage, as evidenced by increased congestion and macrophage infiltration in 3-month PM1-exposed rat lungs. The lung tissue proteomics analysis identified 2673 proteins that highlighted the differential dysregulation of proteins involved in oxidative stress, cellular metabolism, calcium signalling, inflammatory responses, and actin dynamics under exposures to PM1 and gaseous pollutants. The presence of fine particles specifically enhanced oxidative stress and inflammatory reactions under subchronic exposure to traffic-related PM1 and suppressed glucose metabolism and actin cytoskeleton signalling. These factors might lead to repair failure and thus to lung function decline after chronic exposure to traffic-related PM1. A detailed pathogenic mechanism was proposed to depict temporal and dynamic molecular regulations associated with PM1- and gaseous pollutants-induced lung injury.
Conclusion: This study explored several potential molecular features associated with early lung damage in response to traffic-related PM1 or gaseous pollutants, which might be used to screen individuals more susceptible to PM1 air pollution.

P06.01

Dysregulation of Plasma Proteome Induced by SARS-CoV-2 and MERS-CoV Infections Reveal Biomarkers for COVID-19 Patients Disease Outcomes

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Introduction
The global impact of COVID-19 pandemic calls for discovery of biomarkers for effective prognostic stratification of COVID-19 patients. This study aimed to understand the pathophysiology of host responses to infections caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)/ (COVID-19) and Middle East respiratory syndrome coronavirus (MERS-CoV) and to identify proteins for patient stratification with different grades of illness severity.
Method:
Peripheral blood samples from 43 patients with different grades of COVID-19, 7 MERS-CoV patients admitted to the ICU, and 10 healthy subjects were analyzed using label-free quantitative liquid chromatography–mass spectrometry (LC–MS).
Results:
We identified 193 and 91 proteins that differed significantly between COVID-19 and MERS-CoV sample groups, respectively, and 49 overlapped between datasets. Only 10 proteins are diagnostic of asymptomatic cases, 12 are prognostic of recovery from severe illness, and 28 are prognostic of a fatal outcome of COVID-19. These proteins are implicated in virus-specific/related signaling networks. Notable among the top canonical pathways are humoral immunity, inflammation, acute-phase response signaling, liver X receptor/retinoid X receptor (LXR/RXR) activation, coagulation, and the complement system. Furthermore, we confirmed positive viral shedding in 11.76% of 51 additional peripheral blood samples, indicating that caution should be taken to avoid the possible risk of transfusion of infected blood products.
Conclusion:
We identified COVID-19 and MERS-CoV protein panels that have potential as biomarkers and might assist in the prognosis of SARS-CoV-2 infection. The identified markers further our understanding of COVID-19 disease pathophysiology and may have prognostic or therapeutic potential in predicting or managing host cell responses to human COVID-19 and MERS-CoV infections.

P06.02

Proteomic Analysis of the Upper Respiratory Proteins from COVID 19 Patients: A Gel Based Approach.

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Background
COVID-19, a novel acute respiratory syndrome, caused by the SARS-CoV-2 coronavirus, emerged for the first time in Wuhan (China) in December 2019 and quickly it spread across the world as a pandemic ,causing more than 2.7 milion deaths (WHO Coronavirus Dashboard: http://covid19.Who.int). Even if COVID-19 etiology and clinical features are reported in detail by literature, the main biologcal mechanism involved in the pathogenesis of this respiratory syndrome is unknown. Up to date the characterization of all the possible virus-host interactions is mandatory to discover biomarkers to be used for the infectious detection, progression and patient stratification; finally it becomes more and more necessary to develop new diagnostic and therapeutic application.At present RT-PCR represents the current methods of diagnosis of COVID-19 in the biological samples. Previous studies based on targeted approach has been demonstrated that proteomics might represent a poweful tool to analyze the biology of Sars-Cov-2.

Experimental procedures
In the present study we examined the upper respiratory proteome COVID19 negative and positive patients. Once collected all nasopharyngeal swab were tested using qRT-PCR for detecting SARS-Cov-2. Twenty-five were nasal swabs from Covid patients and 12 samples were used from healthy individuals as control. Proteins extracted from the cell pellets were subjected to 2-D gel electrophoresis. The differentially expressed proteins were identified using MALDI-MS analysis.

Results
Identified proteins were classified into different functional groups based on molecular function by Gene Ontology Analysis. An high % of these proteins are cytokeratins involved in cytoskeletal remodellig, whereas only 7% were involved in signal transduction.

Conclusion
This is the first report on respiratory proteome profile in COVID 19 patients by 2DE analysis. Further investigation will be performed comparing the Sars-Cov-2 proteome from nasopharyngeal swabs between subjects infected by the following virus lineage: B.1.1.7 (alpha), P.1 (gamma), B.1.617.2 (delta).

P06.04

Targeted MS Based Multi-Omic Analysis of Blood Plasma from Hospitalized COVID-19 Patients Reveals Predictive Molecular Signatures of Survival

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Introduction
Molecular signatures to discriminate patients based on the risk of severe disease and mortality from COVID-19 infection are urgently required by the global medical community. To date, most COVID-19 biomarker studies have relied on discovery approaches utilizing relative quantitation for the detection of putative biomarkers of infection, severity, and mortality. Although non-targeted methods are useful for comprehensive ‘omic coverage, targeted MS-based approaches generally provide higher precision, and improved inter-laboratory reproducibility, allowing for more realistic materialisation of true biomarkers via validation studies in independent cohorts.

Methods
120 blood plasma samples from 40 hospitalized patients were analyzed by targeted proteomics and metabolomics, utilizing the MRM Proteomics PeptiQuant Biomarker assessment kit and the MolecularYou MYCO 1.1 kit, respectively.

Results
In total, 401 analytes were screened, and the concentrations of 261 analytes were determined. The protein levels of all COVID-19 plasma samples enabled a clear distinction from corresponding reference ranges of healthy plasma samples. PCA-based group comparisons revealed the most significant differences between the survivor and the non-survivor groups. 10 proteins and 13 metabolites that had significantly different (FDR<0.05) concentrations between the two groups, were used to train a support-vector machine classifier model that allowed the prediction of mortality with 83% (proteins), 84% (metabolites), and 90% (proteins+metabolites) accuracy. We applied our model to predict COVID-19 patient mortality on a discovery proteomics dataset from Demichev et al. (MedRxiv prepublished), who identified a 57-protein signature that was predictive of outcomes. We were able to predict mortality with accuracies of 83% and 88%, compared to “not-reported” and 96% in the original study.

Conclusion
Our results suggest that a relatively small subset of molecular features can be used to predict the chances of survival of hospitalized COVID-19 patients within the first day of hospitalization, using a robust LC-MRM setup which is already available in many clinical laboratories.

P06.05

The secretome signature for identifying biomarkers in COVID-19 severe forms

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To face the COVID-19 pandemic caused by SARS-CoV-2 infection that continues spreading worldwide and lead more than 4 million of deaths, many efforts are still being employed to better understand the molecular pathology of the different levels of COVID-19 severity and develop biomarker strategies enabling its early diagnosis. Knowing that the plasma secretome reflects the functional state of cells under pathological evolution, we used a label free mass spectrometry method to define a protein plasma secretome signature of COVID-19 severe forms leading to intensive care unit (ICU) hospitalization and compare the secretome signature to severe pneumonia not from COVID patients also leading to ICU hospitalization. This intrigued us to reveal specific SARS-CoV-2 infection effects on plasma content. For this purpose, the proteome plasma profile of 33 patients hospitalized from severe pneumonia after a SARS-CoV-2 infection, 126 patients hospitalized from severe pneumonia without prior to SARS-CoV-2 infection and 20 control patients were analyzed. The proteome profile was obtained after an enrichment of the low abundant proteins (Proteominer® technology), a trypsin/Lys C digestion and a mass spectrometry analysis by using a NanoAcquity C18 and SYNAPT G2Si mass spectrometer system (Waters) operating in a high definition LC-MSE mode. Based on the quantification of 302 proteins (FDR 1%), we identified 57 significantly deregulated proteins (DEP) in patients hospitalized after a SARS-CoV-2 infection. Several of those DEP are involved in complement activation, platelet degranulation that are known to contribute to the pathogenesis and other biological processes that are less known to contribute to the pathogenesis, such as lipoprotein synthesis. Our approach contributes i) to identify several promising biomarkers for severe forms of SARS-CoV-2 and also ii) to distinguish the molecular alterations induced by SARS-CoV-2 infection.

P06.06

Data-Independent Acquisition Mass Spectrometry (DIA-MS) Analysis Identifies a Neutrophil Proteomic Signature in COVID-19 Infection

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Introduction:
Neutrophils are essential for immune responses to bacterial and viral pathogens but also contribute to immune pathology and tissue injury. Using data independent acquisition (DIA) mass spectrometry we compared the proteome of neutrophils from Hosptalized CoVID-19 patients versus other disease controls and age and sex matched uninfected controls. The objectives were to identify biomarkers that could be used to identify individuals at risk and to identify pathways involved in severe COVID-19 disease.

Methods:
The PREDICT-COVID study was a prospective case control study conducted at a single centre in Scotland, UK. Involving 82 individuals with COVID-19 infection, 91 patients with respiratory infections who tested negative for SARS-CoV-2 (disease controls) and 43 uninfected controls. Neutrophils were isolated from venous blood within 96 hours of hospitalization using negative immunomagnetic selection. The neutrophils were lysed in a SDS lysis buffer and lysates then processed via the S-Trap and analysed on a Q-Exactive HFX mass spectrometer on DIA mode. The DIA data were processed on Spectronaut v14 with subsequent statistical analysis in R.

Results:

The DIA data identified that high levels of interferon induced proteins were a signature of COVID-19 neutrophils, clearly distinct from both infected and uninfected controls. Patient stratification showed no relationship between interferon-induced proteins and either disease severity, or long term outcomes. The stratified analysis detected a proteomic signature that could identify both patients with severe infection, as well as patients with an apparent “mild” COVID-19 infection who subsequently deteriorated.

Conclusions:
COVID-19 infection is associated with profound changes in neutrophil proteomes. This study has identified both potential biomarkers of prognosis, and potential targets for future therapeutic development.

P06.07

Quantitative Proteome and Phosphoproteome Analysis of A549-ACE2 Cells after Infection with Sars-COV2 – A Pilot Study

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Introduction. Coronavirus disease 2019 (COVID-19) is a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), whose outbreak in 2019 led to an ongoing pandemic with devastating consequences for the global economy and human health. According to WHO, COVID-19 has affected more than 162 million people worldwide, with 3,37 million confirmed deaths. Despite the joint efforts of the scientific community there is so much uncharted ground still to cover regarding the mechanisms of SARS-CoV-2 infection and replication. In this perspective, proteomics could help to study these mechanisms.
Methods. In this preliminary study, changes in proteome and phosphoproteome of A549 pulmonary epithelial cells transfected with the ACE2 (A549-ACE2) were analyzed 24h after infection with SARS-CoV-2. We have used a TMT-based proteomics quantitative approach and TiO_2 phosphopeptide enrichment.
Results. A total of 4304 protein groups were identified (FDR<1%), of which 121 were found differentially regulated in the SARS-CoV2 compared to MOCK-infected cells (q-value<0.05). Label-free quantitative analysis after TiO_2 enrichment allowed the identification of 2458 protein groups (more than 5000 phospho-sites), of which 309 were found differentially regulated (q-value <0.05). Cellular processes such as phagosome maturation, glucocorticoid receptor signaling, inflammation signaling, and innate immunity were found altered according to IPA analysis. Some of them were found related to upstream regulators such as transcriptional factors (PPARA), inflammatory regulators (TNF, IFNG, CSF1), proliferation factors (MAPK), and cholesterol biosynthesis regulators (INSIG1, SCAP, SREBF2). Phosphorylated proteins were found mainly associated with mRNA translation and splicing, cell proliferation and apoptosis (HIPPO Signaling), cell cycle regulation, protein ubiquitination, among others. Estrogen receptor, heat shock factors (HSF1), transcriptional factors (E2F4, YAP1, CEBPB), and tumor suppressor gene (MLH1) were found as potential upstream regulators.
Conclusion. Although these data are preliminary, these results clearly explain some of the mechanisms associated with infection of human cells by SARS-COV2.

P06.08

Glycopeptide Mapping for Comparison of CHO and HEK Cell Derived SARS-Cov-2 Spike Trimeric Protein Antigen

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Introduction
SARS-Cov-2 Spike trimeric protein for serological testing has been produced in CHO cells. CHO cells have human-compatible glycosylation capability, but their N glycan profile (182 glycans) is in part different to human (39 glycans). The glycan shield composition of SARS-CoV-2 spike protein affects potential development of therapeutic products (2). Thus our CHO derived His- tagged full length protein requires characterisation and comparison with human derived equivalent to assess clinical utility.

Methods
Recombinant Spike protein derived from a HEK293 or CHO expression system (1) was subjected to proteolytic digestion by either trypsin or Glu-C. Peptides were analysed by LC-MS/MS using a QExactive -HF instrument. Mass spectra were acquired with automated data-dependent switching between full-MS and tandem MS/MS HCD scans using stepped collision energy. Data analysis was performed using peptide-mapping tool of BioPharma Finder 4.0 using integral human and CHO specific N glycan libraries and O glycan database.

Results
22 N glycosylation sites were assigned for both HEK and CHO derived SARS-Cov-2 Spike protein. Trypsin and Glu-C yielded glycopeptides, providing complementarity for CHO Spike protein. 8 potential O glycosylation were identified of which 5 were common to HEK and CHO. In term of variety and range of glycans, 43 and 137 were identified for HEK and CHO respectively.

Conclusions
Cell type specific glycosylation analysis was achieved. Complex and oligomannose and hybrid N glycans were identified for CHO. Similarities and differences were identified between the CHO derived Spike protein relative to HEK. A key difference was that tri and tetra antennary structures were found only in CHO, consistent with the literature that CHO has more high branched complex patterns, Levels of sialylation were higher in CHO than HEK, with fucosylation at similar levels. These data have can inform the value of CHO derived Spike protein beyond utility in serological testing.

P06.09

Sex differences in Autoantibodies Response to SARS-CoV-2 Infection

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Introduction: Emerging evidence has highlighted the importance of autoimmune activation in modulating acute responses and recovery trajectories following SARS-CoV-2 exposure. The aim of the study was to assess autoimmune activation after COVID-19 illness in the absence of comorbidities, via protein array detecting autoantibodies (AABs) to over 90 antigens associated with classic autoimmune diseases. Secondly, we assessed the correlation between sex and AAB response titer, and symptoms and AAB response titer.

Methods: 177 plasma samples obtained from SARS-CoV-2 positive individuals based on presence of positive anti-nucleocapsid IgG serology results (Abbott Diagnostics) and 53 plasma sample from pre-pandemic and healthy individuals were analyzed using a bead-based protein assay featuring 91 autoantigens. Multivariate analysis was implemented to assess sex-specific AAB titers with respect to results from a symptom questionnaire.

Results: AAB reactivity and symptom frequency were shown to be sex specific in SARS-CoV2 infection. We found a distinct set of AABs to 59 antigens highly correlated with reported symptoms in the male population, while another set of AABs to 38 antigens were associated with symptoms in females. The high frequency associated AABs included SNRPB, a ribonucleoprotein widely prevalent in human systemic lupus erythematosus. The moderate frequency associated AABs included MOV10, CHD4, HIST1H4A, ACE2, IFNA6, LYZ, RNF41. The most prominent symptoms in females were associated with AABs to DBT and ROS1. We observed these sex-specific AAB associations up to 6 months following symptomatology, indicating that SARS-CoV-2 triggers a complement of AABs responses that persists over time irrespective of illness severity.

Conclusion: Our findings underscore the serological diversity underlying the clinical heterogeneity of COVID-19 infection and its sequelae, including the long-COVID phenotypes. Males have an increased risk of a more diverse autoimmune response following symptomatic COVID-19 illness, while females are associated with a different profile of autoimmune activation following asymptomatic SARS-CoV-2 exposure.

P06.10

SARS-CoV-2 Infection Triggers Auto-Immune Response in ARDS

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Acute respiratory distress syndrome (ARDS) is a severe pulmonary disease which is one of the major complications in the COVID-19 patients. Dysregulation of the immune system and an imbalance in cytokine release and immune cell activation are involve in SARS-CoV-2 infection. Here, it has been analyzed inflammatory, infectious antigen and auto-immune profile of patients presenting severe ARDS in the course of COVID-19 disease using functional proteomics. Both, innate and adaptative immune humoral response have been characterized through acute-phase protein network and auto-antibody signature. Severity and sepsis infection by SARS-CoV-2 seem to be correlated with auto-immune features of patients and determined their clinical progression; which could provide novel perspective in therapeutics and biomarkers of COVID-19 patients.

P06.11

Utilisation of Cyclic Ion Mobility with Multiple Pass Acquisition for the Analysis of Glycopeptides and Glycoforms Associated with SARS-CoV-2

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Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to be responsible for the large-scale epidemic globally. The SARS-CoV-2 S protein is highly conserved and involved in multiple processes, including receptor recognition and viral attachment. The viral S protein is modified by glycosylation which may be implicated in immune evasion from the host immune system by shielding the protein surface from detection by antibodies, affecting the ability of the host to mount an effective adaptive immune response. It has therefore become an important target for vaccine research. Here, we demonstrate the utility of Cyclic IMS (cIMS) for in-depth glycopeptide characterisation using the multi-pass feature to separate co-eluting glycoforms related to the SARS-CoV-2 S1 protein.

Methods and Results

Initial assessment of the digest was conducted using HDMSE, which comprised of a single pass of the cyclic device. The resulting data were processed and searched against a sequence specific database. Based on the initial, single pass data and observation of typical oxonium ions within the fragment ion spectra, glycopeptides at m/z 969.08 (3+) and 1262.9 (3+) were selected as candidates for further investigation using quadrupole isolation and multi-pass ion mobility. In order to allow multi-pass acquisitions, the cIMS settings were configured with mobility times derived from the cIMS pass calculator and by measuring the ion behaviours for 1 and 2 passes. A total of five passes of the cyclic device was sufficient to show multiple glycoforms for the ion at m/z 1262.9 and it was found that the fragment spectra corresponding to this species had the characteristic sialic acid linkage (m/z 657.2). Interpretation of the glycopeptide sequences was conducted using the GlycReSoft, in addition to manual interpretation.

Conclusions

Implementing the Cyclic IMS with multi-pass acquisition provides glycopeptide identifications with separation of individual and potentially unique glycoforms.

P06.12

Longitudinal Proteomic Profiling of Dialysis Patients with COVID-19 Reveals Markers of Severity and Predictors of Death

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Introduction: End-stage kidney disease (ESKD) patients are at high risk of severe COVID-19. Despite this, ESKD patients cannot shield as they must continue to receive haemodialysis treatment. We leveraged the unique opportunity for longitudinal sampling from early symptoms through hospitalisation afforded by the largest haemodialysis cohort in the UK. We employed Olink proteomics to characterise the temporal dynamics of the proteome following COVID-19 infection.

Methods: We performed serial blood sampling in ESKD patients with COVID-19 (256 samples from 55 patients). We also recruited 51 non-infected ESKD patients as controls. A further 46 COVID-19 patients were enrolled to form a validation cohort. We used Olink immunoassays to measure 436 circulating proteins. Linear mixed models were used to perform differential expression analysis between cases and controls and to identify proteins associated with disease severity within COVID-19 patients. We used random forests to predict COVID-19 severity and extract important biomarkers. Longitudinal analysis was performed using linear mixed models and survival analysis.

Results: Comparison of COVID-19 positive and negative patients revealed 221 differentially expressed proteins, with consistent results in a separate subcohort. Two hundred and three proteins were associated with clinical severity, including IL6, markers of monocyte recruitment (e.g. CCL2, CCL7), neutrophil activation (e.g. proteinase-3), and epithelial injury (e.g. KRT19). Machine-learning identified predictors of severity, including IL18BP, CTSD, GDF15, and KRT19. Survival analysis with joint models revealed 69 predictors of death. Longitudinal modelling with linear mixed models uncovered 32 proteins displaying different temporal profiles in severe versus non-severe disease, including integrins and adhesion molecules.

Conclusions: These data implicate epithelial damage, innate immune activation, and leucocyte–endothelial interactions in the pathology of severe COVID-19 and provide a resource for identifying drug targets.

P06.13

Large-Scale Discovery and Exploration of Virus-Host Interaction Motifs

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Introduction: Short Linear Motifs (SLiMs) are 3-10 amino acid long stretches of protein sequences predominantly found in intrinsically disordered regions. They serve as docking sites and are recognized by globular protein domains of interactors. SLiM-based interactions are crucial for cellular signaling, trafficking, and translation regulation. Viruses, including SARS-CoV-2, exploit SLiM-based interactions to hijack host pathways, promote viral survival, replication and egress. We have generated a pipeline for the large-scale charting of how viruses employ SLiM-based interactions and exploring how the information can be used to identify novel targets for developing antiviral agents.

Methods: Our systems biology approach to chart the SLiM-based host-pathogen interactions on large scale included i) curation of literature on viral SLiMs into a new database, ii) generation of a phage peptidome[1] representing the disorderome of 229 RNA-viruses and screening it against 139 human bait proteins, and iii) validation of selected cases. Validations through biophysical and structural approaches and affinity purification-mass spectrometry (AP-MS) were focused on three processes, (i) ESCRT pathway (ii) clathrin-mediated trafficking pathway, and (iii) PABP-mediated translation regulation.

Results: Manual literature curation generated a database of more than 900 experimentally validated viral SLiMs. Through phage display screening we identified more than 2,000 SLiM-based viral interactions with about 100 human proteins. We uncovered several viral species exploiting the ESCRT pathway for viral egress. Moreover, we propose clathrin-mediated trafficking as a hub of viral interference. Finally, we identified PABP as a viral target. Structural details of the complexes are provided via co-crystallization while through AP-MS the specificity of the viral SLiM-based interactions was validated.

Conclusion: We more than doubled the available information on viral SLiM mimicry and contributed to a global perspective of host-pathogen interactions. The detailed information on viral binding sites may lead to the identification of novel druggable targets.

Reference:

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P06.14

Antibody Landscape against SARS-CoV-2 Proteome Revealed Significant Differences between Non-structural/ Accessory Proteins and Structural Proteins.

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·Introduction
One of the major features of patients with COVID-19 is the extreme variability of clinical severity from asymptom to death. However, the factors that cause this variability, and the immunogenicity of SARS-CoV-2 proteome are largely unknown, especially for non-structural proteins and accessory proteins, the prevalence, clinical relevance and the dynamic of which in patients are still not clear.
·Methods
Here we collected 2,360 COVID-19 sera and 601 control sera. We analyzed these sera on a protein microarray with 20 proteins of SARS-CoV-2, built an antibody response landscape for IgG and IgM.
·Results
We found that non-structural proteins and accessory proteins NSP1, NSP7, NSP8, RdRp, ORF3b and ORF9b elicit prevalent IgG responses. The IgG patterns and dynamic of non-structural/ accessory proteins are different from that of S and N protein. The IgG responses against these 6 proteins are associated with disease severity and clinical outcome and declined sharply about 20 days after symptom onset. In non-survivors, sharp decrease of IgG antibodies against S1 and N protein before death was observed.
·Conclusion
Using a SARS-CoV-2 proteome microarray, we found 6 non-structural/accessory proteins elicit strong antibody responses in patients with COVID-19. The global antibody responses to non-structural/ accessory proteins revealed here may facilitate deeper understanding of SARS-CoV-2 immunology.
·Reference:
Li et al., Antibody landscape against SARS-CoV-2 proteome reveals significant differences between non-structural/ accessory proteins structural proteins.Cell Rep . 2021 Jul 13;36(2):109391.
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P06.15

Multi-omics Characterization of COVID-19 Reveals Risk Factors for One-year Sequelae

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Introduction
Over 160 million individuals have recovered from COVID-19 but little is known about the risk of sequelae.
Methods
We comprehensively assessed clinical and multi-omics (proteomics and metabolomics) characteristics of 144 COVID-19 patients with up to 397-days follow-up.
Results
We found that serum CALCOCO2 was down-regulated since the disease onset and remained suppressed at the one-year follow-up. Patients with severe symptoms, elder ages, comorbidities, elevated serum urea and decreased estimated glomerular filtration rate (eGFR) were more prone to suffer from lung or kidney sequelae. Our data showed urinary protein changes during acute phase associated with kidney sequelae. Finally, machine learning associated sequelae with 20 serum proteins, nine urine proteins, seven metabolites, and nine clinical indicators. The levels of these risk factors measured during the first month of COVID-19 predicted one-year sequelae with an accuracy of 87.5%.
Conclusions
This resource could enable multi-dimensional interpretations for the understandings of COVID-19 immunological characteristics and post discharge sequelae.

P06.19

Systematically Exploit the IgG Responses to SARS-CoV-2 at Amino Acid Level by AbMap

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Introduction:
COVID-19 is a worldwide pandemic caused by SARS-CoV-2. By July 22, 2021, more than 190 million cases were diagnosed, and more than 4 million deaths were claimed (https://coronavirus.jhu.edu/map.html). At present, more and more mutant strains of SARS-CoV-2 emerged and spread fast. To develop antibodies or vaccines precisely for these variants, given the small size of binding regions, it was necessary to systematically exploit the IgG responses to SARS-CoV-2 at amino acid level.

Methods:
A set of 55 convalescent sera and 226 protein/ peptide enriched antibodies (obtained from above convalescent sera in a consequential manner) were dissected by AbMap, which was a method for high-throughput epitope mapping. At last, some epitopes were validated by peptide microarray.

Results:
From the convalescent sera, we have identified 418 motifs, 275 of which could be matched to 27 of the 28 known SARS-CoV-2 proteins. More motifs were identified in the protein/ peptide enriched antibodies. After plotted the epitopes and the frequencies alongside the linear sequence and domains of S protein or N protein, two hot areas (one almost covered the entire CTD, and the other covered the S2’ protease cleavage site and the fusion peptide) in S protein and one in N protein were identified. In the validation experiments, for all the three selected epitopes, when the corresponding samples were tested, significant binding signal loss was observed when any of the critical residue was mutated to alanine.

Conclusions:
These results facilitate the in-depth understanding of SARS-CoV-2 specific IgG responses, provide hints for precise development of diagnostic reagents, therapeutic antibodies and even vaccines.

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P06.20

A COVID-19 Knowledge Graph for Therapeutic Discovery from Semantic Integration of Literature and Databases

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Introduction: In response to the COVID-19 global health emergency, the COVID-19 literature is rapidly expanding. Computational approaches that automatically distill key information from text and integrate it with information from curated biological databases are essential to gain insight into COVID-19 etiology, diagnosis and treatment. Knowledge graphs (KGs) are a powerful way to represent such diverse biological information and generate novel hypotheses. In this study, we constructed a COVID-19 knowledge graph based on mining of literature and databases, using semantic web technologies (RDF and SPARQL) for data integration.
Methods: The KG integrates information extracted from (i) the COVID-19 literature using the text-mining tools iTextMine (PTM and miRNA relations), PubTator (biomedical entities), and SemRep (biomedical relations based on UMLS); (ii) curated databases, such as UniProtKB and DrugBank; and (iii) proteomic and phospho-proteomic data on SARS-CoV-2-infected cells. It is served by the OpenLink Virtuoso server community edition with SPARQL 1.1 query federation.
Results: The COVID-19 KG, consisting of 22 named graphs and 1.2 billion RDF triples, is accessible via a knowledge portal (https://research.bioinformatics.udel.edu/covid19kg/) with browsing and search interfaces; YASGUI (Yet Another Sparql GUI) with a set of comprehensive SPARQL queries for new users; and a RESTful API. Using the KG, we identified several potentially beneficial COVID-19 therapeutics, including drugs targeting TNF and IFN-gamma, two proteins implicated in the cytokine storm that affects some patients with severe COVID-19, as well as kinase inhibitors and miRNAs that may disrupt key molecular interactions of the SARS coronavirus nucleocapsid protein, a heavily phosphorylated protein required for viral genome replication and packaging.
Conclusions: With its unique focus on molecular relations, ability to keep up with the latest published results via text mining, and inclusion of a wide variety of biomedical knowledge using a semantic framework, our KG can provide insight into the rapidly evolving landscape of COVID-19.

P06.22

Systematical Deciphering of SARS-CoV-2 Specific Humoral Immune Responses

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Introduction
The global pandemic of COVID-19 is caused by SARS-CoV-2. As of July 26, there are 194 millions of diagnosed cases and >4 millions of death are reported. The pandemic is still unfolding. SARS-CoV-2 specific antibodies, especially the neutralizing antibodies, play key roles during infection and recovery. It is of great interest to decipher how the SARS-CoV-2 specific antibodies are generated and also their dynamics.

Methods
To decipher the SARS-CoV-2 specific antibody responses, we have developed three platforms. The first platform is a SARS-CoV-2 proteome microarray, which carries 21 of the 28 predicted proteins, the second platform is a peptide microarray with 197 12-mer peptides which entirely cover the Spike protein, the third is a high-throughput techniques (AbMap) for mapping the antibody binding epitopes.

Results
Over 3,000 sera collected from >1,000 COVID-19 patients, and >600 control sera were analyzed by these three platforms. SARS-CoV-2 specific antibody responses were revealed. Correlations among antibody responses, clinical parameters and disease severity were identified. Biomarkers for a variety of purposes were determined.

Conclusions
We have constructed a comprehensive map of SARS-CoV-2 specific antibody responses at three levels, i. e., protein, peptide and single amino acid. The map will help us for better understanding of the SARS-CoV-2 triggered humoral immune responses, identifying biomarker for diagnostics, as well as the precise development of therapeutic antibodies and vaccines.

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P07.01

Saliva Protein Signatures of Smokers Enrolled in Lung Cancer Screening for Early Diagnosis and Clinical Management.

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Background
Lung cancer (LC) neoplasm with the highest incidence (both genders, smokers and non-smokers) and mortality rate worldwide, constitute as adenocarcinomas a biologically heterogeneous group, Non-Small Cell Lung Cancer (NSCLC) that accounts for 85% of all LC; although progress has been made in diagnosis and teraphy, the prognosis of non-small-cell lung cancer (NSCLC) is poor, with a 5-year survival that decrease at late developmental stage (4°) up to 10%. Only 15% of NSCLC are detected at early stage and Low Dose LDCT is used as screening method for LC in high-risk subjects, 55-85 age old with 30-year smoking history and who have not quit for > 15 years. Even though screening with LDCT reduced LC mortality by 20% compared to chest X-rays, LDCT scans had a false positive rate (FPR) > 95% (2). It is necessary a screening test for LC with a lower FPR. Saliva like all diagnostic fluids give some information useful for early disease detection, disease prognosis and risk stratification monitoring treatment response.
Experimental procedures At first we developed a standardized sample preparation method for undoubt identification of molecolar targets, unique, useful for clinical and therapeutical purpose collecting salivary fluid from 140 volunters enrolled for LASMOT SCREENING project, all heavy smokers, 55-75 age old. All persons subjected to LDCT were grouped in 83 negative controls without nodular lesion and 57 positive for nodular or pseudonodular lesion. Results. Comparing saliva proteome between negative and positive samples we identified 44 costituvely salivary proteins in LDTC negative smokers and 23 newely salivary proteome in LDTC positive smokers.
Conclusion. We found in LDTC positive differentially expressed a S100A14 linked to cellular events related to carcinogenesis. It may predict a poorer survival. So “Gel proteomics" study on salivary aimed at identifying molecular targets released at the initial stage of smoking-related pulmonary cancegerogenesis

P07.02

Connecting Molecular Pathology and Precision Oncology: Development and Validation of a Quantitative Immuno-MRB Assay for The PD-1/PD-L1 Axis

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Introduction
Therapeutic antibodies that target checkpoint inhibitors (CPIs) like PD-L1 and restore anti-tumour immune response have revolutionized lung cancer treatment. Patients are selected based on PD L1 immunohistochemistry (IHC), which is notoriously heterogeneous and can be affected by tissue fixation time and post-translational modifications. Consequently, ~50% of patients with high PD-L1 levels do not respond to CPI, while some patients with low/undetectable PD-L1 do. Multiple studies indicate that PD-L1-expression alone does not reliably reflect the tumour microenvironment, thus necessitating the measurement of other members of the PD-1/PD-L1 signalling pathway. We, therefore, developed a multiplexed immuno-multiple reaction monitoring (iMRM) assay for quantifying six proteins of the PD-1/PD-L1 axis.

Methods
Anti-peptide antibodies were generated against selected proteotypic peptides of PD L1, PD-1, PD-L2, NT5E, LCK, ZAP70. LC, MRM, and anti-peptide immunoprecipitation (IP) parameters were optimized to improve linear range, lower limit of quantitation, recovery, and reproducibility for 13 targeted peptides. We analyzed 19 non-small cell lung cancer (NSCLC) FFPE cores of 1-2 mm^3 from tumours with PD-L1 IHC staining ranging from negative to high using our iMRM workflow.

Results
Based on CPTAC guidelines, our LC-MRM method allows the quantitation of PD-L1 and PD-1 down to 21 amol on-column. The average IP-recovery was 83±2%. PD-L1 expression in the 19 NSCLC tumours was 8-631 amol/µg of total protein and only weakly correlated (R^2=0.404) with PD-L1 IHC. Unsupervised hierarchical clustering of our iMRM data yielded two “low-expression/poor-prognosis” and “high-expression/good-prognosis” groups, with 66 months and 111 months of average survival, respectively.

Conclusion
We developed a robust iMRM workflow for the quantitation of the PD-1/PD-L1 axis from FFPE tissues. Our proof-of-concept data show great promise for clinical utility. Assays to determine the glycosylation status of PD-1, PD-L1, and PD-L2 are currently being added with the goal to further explain the discrepancy between IHC results and patient response.

P07.03

Extracellular Vesicle Protein Biomarkers of Cardiac AL Amyloidosis

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Amyloidosis is characterized by extracellular deposition of insoluble fibrils formed from the aggregation of misfolded autologous proteins, resulting in tissue structure disruption and organ dysfunction. Systemic light-chain (AL) amyloidosis is one of the most common form. It is due to over-production of immunoglobulin free light chains, most frequently caused by a plasma cell clonal proliferation. The diagnosis currently relies on analysis of a tissue biopsy and is often made late in the course of the disease, when patients already suffer from severe complications linked to cardiac involvement. Therefore, the identification of new biomarkers in the plasma or serum is needed to facilitate early recognition of AL amyloidosis. As it is known that the molecular content (including proteins) released in extracellular vesicles (EVs) reflects the physio-pathological state of an organism, we evaluated the interest of using the protein EV content from plasma to identify protein biomarkers of AL amyloidosis. After isolation by ultrafiltration of EVs from the plasma of AL amyloidosis patients (n=9) and patients with ischemic heart disease (n=12), we performed a label-free quantitative proteomic approach with a trypsin/Lys C digestion and a high definition LC-MSE mass spectrometry analysis by using a NanoAcquity C18 and SYNAPT G2Si mass spectrometer system (Waters). The protein identification and quantification were performed by using Progenesis for proteomics software (Waters). We quantified 266 proteins with a FDR 1% and identified 12 significantly deregulated proteins (DEP) in AL amyloidosis patients, which should be further studied as candidate biomarkers. Interestingly, several of them are involved in complement regulation and platelet degranulation that are known to contribute to amyloidosis pathogenesis. This confirms the interest of studying the protein content of plasma EV to identify AL amyloidosis biomarkers.

P07.05

Phospho-Proteome Analysis of Cerebrospinal Fluid Extracellular Vesicles in Primary Central Nervous System Lymphoma

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Primary central nervous system lymphoma(PCNSL) is a rare extra-nodal non-Hodgkin's lymphoma and accounts for 3%-4% of central nervous system tumors. Recent studies have highlighted the importance of cerebrospinal fluid derived extracellular vesicles in PCNSL. However, studies of CSF EVs are mainly limited by the amount of EVs isolated from per milliliter of CSF and the volume of CSF acquired from one patient. Here, we provide a label-free quantitative phospho-proteome profiling of EVs separated from PCNSL and non-PCNSL CSF samples by an earlier introduced functional magnetic beads called EVTRAP together with highly sensitive timsTOF Pro.

EVs were isolated by EVTRAP magnetic beads from 3mL pooled CSF and 99% of the sample was followed by an additional phosphopeptide enrichment using polyMAC, prior to LC-MS/MS analyses. The raw files were searched using PEAKS Studio X+ software. The false discovery rates (FDRs) of proteins, peptides, and phosphosites were all set to 1% (-10lgP ≥ 20 ≥1 unique peptide for proteins). For label-free quantification of both proteome and phospho-proteome, data were normalized using Total Ion Current (TIC) signals and between-group comparisons were analyzed by one-way ANOVA.

A total of 1049 phosphoproteins, 5470 phosphopeptides were identified in non-PCNSL group and 1232 phosphoproteins, 6567 phosphopeptides were identified in PCNSL group. Furthermore, intra group reproducibility of phosphoproteins was over 50%. Besides, several PCNSL-related pathways and proteins were found.

A considerable amount of phosphoproteins and phosphopeptides are identified from about 2.1mL CSF through a highly efficient EV capture beads named EVTRAP combined with highly sensitive timsTOF Pro, which can help promote researches in CSF EVs.

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P07.06

Mapping Isoform Abundance and Interactome of the Endogenous TMPRSS2-ERG Fusion Protein in Prostate Cancer

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Introduction
We previously demonstrated numerous applications of quantitative proteomics for discovery and validation of prostate cancer and male infertility biomarkers [1]. Here, we focused on TMPRSS2-ERG fusion, a genomic alteration found in ~50% of primary prostate cancers. While this fusion has been extensively characterized at mRNA level, identity of an endogenous fusion protein and its isoforms has never been resolved at the protein level.

Methods
We developed highly sensitive immunoaffinity (IA)-mass spectrometry assays for quantification of a low-abundance endogenous TMPRSS2-ERG fusion protein, its isoforms and its interactome. Orthogonal immunoprecipitation with N-term and C-term antibodies provided differential enrichment of two isoform groups, and selected reaction monitoring (SRM) assays unambiguously resolved and quantified each distinct isoform in prostate cancer VCaP cells and formalin-fixed paraffin-embedded (FFPE) tissues.

Results
We quantified endogenous TMPRSS2-ERG fusion protein (~27,000 copies/per VCaP cell), discovered its four distinct isoforms, and revealed that T1E4-ERG isoform accounted for 52% of total TMPRSS2-ERG protein in VCaP cells, and 50% in prostate cancer FFPEs. Sensitivity of our assay was sufficient to differentiate fusion-positive and -negative FFPEs, and the results agreed with our in-house immunoassay data. For the first time, a unique N-terminal peptide of TMPRSS2-ERG fusion (M-truncated and N-acetylated TASSSSDYGQTSK) was identified. Interactome of the endogenous TMPRSS2-ERG revealed numerous transcriptional regulators, including mutually exclusive BRG1- and BRM-associated SWI/SNF chromatin remodeling complexes [3].

Conclusions
Our sensitive and selective IA-SRM assays present novel tools to measure TMPRSS2-ERG protein and its distinct isoforms in prostate tissues and cells, and will facilitate development of precision diagnostics of prostate cancer subtypes.

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P07.08

Proteomic Profiling of Ankylosing Spondylitis Patients Serum Reveals Biomarkers for Therapeutic Response Prediction and Associated Mechanistic Insights

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Introduction: Radiographic axial Spondyloarthritis (r-axSpA), the prototypical form of axial spondyloarthritis, can lead to significant disability and impairment in quality of life. Effective therapy delay for non-responders imply continued impact of disease. Adalimumab, is a disease-modifying antirheumatic drug and monoclonal antibody that works as an immunosuppressive medication by inactivating TNF-alpha.
Methods:Proteomic analysis involved 33 patients with r-axSpA (19 responders and 14 non-responders) during 14 weeks treatment with adalimumab. Serum samples were collected at baseline (BL), 3-5 days (D3), 2 (W2) and 14 weeks (W14) after treatment. Response to adalimumab was defined as the achievement of ASAS20. LC-MS/MS protein levels were submitted to multivariate, univariate and ROC analysis.
Results: 333 proteins were identified with at least 2 non-ambiguous peptides. Two sets of 5 proteins were identified displaying differences between responders and non-responders ($p < 0.05$) at BL and D3. Four differential proteins at D3 confirmed to be predictive of response to treatment (ROC AUC, sensitivity and specificity of 0,98, 88% and 100%, respectively). C-reactive protein, an inflammation marker, lowered in the plasma at W2 in both responders and non-responders. Moreover, in responders a protein cluster associated with plasma lipid particles involved in lipid transport increased at W14, while in NR it started at W2. An increment was found for proteins associated with the complement system activation in innate defense with the responders presenting the earlier reaction. An augmentation of proteins involved in the insulin-like growth-factor system was shown at W14 only for responders, suggesting a stimulation of cartilage cells protection and osteocytes activation that could constitute an anabolic factor for bone.
Conclusions: Taken together, our results suggest novel biomarkers to evaluate the potential response to adalimumab a few days after initiating treatment. In responders, adalimumab treatment seems to promote normal bone and tissue growth and development.

P07.11

Cancer-Testis Antigen and Immune Profiling in Non-Small Cell Lung Cancer by Transcriptomics and Antibody-Based Proteomics

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Introduction
Immunotherapy has revolutionized the treatment of lung cancer. However, only a minority of patients show long-term benefit. Accumulating evidence indicates that the antigenic repertoire of tumors is critical for a successful anticancer immune response. Physiologically expressed in testis, cancer-testis antigens (CTAs) are ectopically expressed in lung cancer and known to elicit a humoral and cellular immune reaction. The study aimed to characterize CTA expression in non-small cell lung cancer (NSCLC) in the context of the immune microenvironment and clinical outcome.

Methods
Of 90 CTAs previously validated by RNA expression, eight (DPEP3, EZHIP, MAGEA4, MAGEB2, MAGEC2, PAGE1, PRAME, and TKTL1) were selected for immunohistochemistry on tissues from 328 NSCLC patients based on expression pattern and previous literature. Additionally, eleven immune markers were used to assess the immune repertoire of NSCLC. The immunohistochemical data was further compared with mutational and RNA-seq profiles, as well as clinical parameters.

Results
A majority of NSCLC cases (79%) expressed at least one of the analyzed CTAs and protein expression correlated in general with RNA expression. Interestingly, the eight selected CTAs had different expression profiles, with MAGEA4 predominantly expressed in adenocarcinoma and PRAME in squamous cell carcinoma. High PAGE1 or EZHIP expression were associated with higher plasma cell infiltration, and high TKTL1 expression correlated with lower PD-L1 expression (p-adj <0.05, all comparisons). Global RNA-seq analysis of tumors with high or low CTA protein expression identified differentially expressed genes, including other CTAs and immune-related genes. CTA protein expression was not associated with mutational status, performance status, or survival.

Conclusions
The current study provides a comprehensive evaluation of known and uncharacterized CTAs in NSCLC. The association of CTAs with specific immune cells indicates an in-situ immunogenic effect and provides the basis for focused evaluation. The findings also support the rationale to harness CTAs as targets for immunotherapy.

P07.12

Cytokines and Chemokines Analysis of Malignant Pleural Effusions

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Introduction: MPE is clinically common in advanced-stage cancer patients and associated with worse survival. Based on previous studies, we hypothesized two distinct immune phenotypes of MPEs with different prognoses. This study aimed to explore the cytokines/chemokines biomarkers and potential interactions between two immune phenotypes of MPEs.

Methods: The human proteome profiler arrays for cytokine and chemokine were performed and analyzed qualitatively and quantitatively using ImageJ/Fiji software. Selected cytokines and chemokines were further quantified with enzyme-linked immunosorbent assays (ELISAs).

Results: 42 cytokines and 12 chemokines were both detected in MPEs with two immune phenotypes. The cytokine CD30, CD26, IGFBP-3, MIF, VEGF, and the chemokine CXCL8, Midkine, CCL19, CXCL4 indicated a larger value for fold change of protein expression than in MPE with a better prognosis. The angiogenic factor VEGF-A by ELISA indicated more than 2-fold higher levels in MPE with poorer prognosis and demonstrated a pro-angiogenesis phenotype.

Conclusions: The pleural cytokine/chemokine profile supported the hypothesized two distinct immune phenotypes of MPEs with different prognoses. The significantly increased VEGF-A indicated tumor-promoting angiogenesis in MPE associated with poorer survival. Targeting tumor angiogenesis may present a promising strategy for MPE patients with worse prognoses.

P07.13

Comprehensive Serum Proteome Analysis for Signatures Development in High-Grade Serous Ovarian Cancer

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Introduction: Ovarian cancer is the most common cause of death among woman in Korea. Though the 5-year survival rate of ovarian cancer is approximately 62.1% which is improved up to 90% diagnosed in early stage. It raises a need for biomarkers because there are no symptoms in the early stage and tissue collection is difficult without using invasive methods.
Methods: A total of 55 patients were diagnosed with high-grade serous ovarian cancer (HGSOC). For a comparative study, 49 healthy control subjects (HC) were applied. Individual serum proteome was characterized using data-independent acquisition (DIA) label-free LC-MS. The DIA data was interrogated using a comprehensive spectral library created by deep-proteome profiling with online 2D-NCFC-RP/RPLC system from the same serum samples.
Results: Initial characterization experiments using unbiased DDA coupled with online fractionation system facilitated the building of human serum protein spectral library. A total of 90,947 non-redundant peptides covering 4,208 genes were observed in the library. The spectral library information took reference information to perform qualitative and quantitative determination for individual sample DIA analyzed. For proteome analysis, more than 30,000 peptides, 977 proteins were quantified at FDR < 0.05 across 104 serum samples. Proteins and peptides detected more than 50% at each group were taken for statistical comparison, i) Student t-test p-value, ii) Wilcoxon-Ranksum test p-value, iii) Stouff's p-value combination, and iv) fold-change. Differentially expressed proteins (DEPs) between HGSOC and HC at protein and/or peptide levels were mapping to KEGG pathway database. Finally, DEPs identified here and those correlated with the immune response and cellular growth might represent candidate biomarkers.
Conclusions: Further validation using multiple reaction monitoring (MRM)-MS alternative approach and eventually functional studies, are in progress. Selected biomarker candidates, however, can be used as baseline data for the development of clinically usable biomarker of HGSOC.

P07.14

Diagnostic Value of Multiple Serum Protein Marker in Breast Cancer Based on Proteomics Technique

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Introduction: Breast cancer is the highest incidence of cancer among women in the world, and there is a need for more accurate and objective diagnostic methods as there are deviations in test methods and results for diagnosis. In this study, we assess the adequacy and reproducibility of breast cancer diagnosis for multiple blood markers (Mastocheck®) developed with proteomics techniques in previous studies.
Methods: Using Multiple Reaction Monitoring (MRM), one of the analysis techniques of the mass spectrometer, the concentration of the target proteins of Mastocheck® is analyzed. Since then, we have developed an early breast cancer screening model (algorithm) using logistic regression to match the concentration values of the three proteins measured in that range. In parallel, new blood samples not used in the development of the algorithm were prospectively or retrospectively collected analyzed for validation studies.
Result: A total of 1,469 blood samples were analyzed, pooling the blood samples that analyzed with the final algorithm. There are 824 histologically confirmed breast cancer and 645 healthy blood samples. Sensitivity, specificity, and accuracy for breast cancer diagnosis were 82.1%, 70.4%, and 76.3%, respectively. Patients with stage 0-4 breast cancer were enrolled, and patient's individual characteristics (age, blood pressure, diabetes, hyperlipidemia) were not specifically correlated with blood marker values.
Conclusion: Mastocheck® is the first KFDA (Korean Food and Drug Administration) approved biomarker for in vitro diagnosis of breast cancer. The results of this study show that Mastocheck® has enough performance and reproducibility for diagnosis in breast cancer. Further studies are under way to demonstrate diagnostic value and prognostic performance.

P07.15

The Effect of Storage Time and Temperature on MS Analysis of FFPE Tissue Sections

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Introduction: Formalin-fixed paraffin-embedded (FFPE) tissues present an invaluable resource for cancer proteomics. They are more readily available than fresh frozen tissues because they can be stored at ambient temperature for decades. However, immunohistochemistry (IHC) studies suggest some protein antigenicity can degrade over time in FFPE sections. It is not known whether FFPE sections used for LC-MS analysis are affected by storage time or temperature. We determined the stability of FFPE sections stored at room temperature (RT) versus -80°C over 336 days.
Methods: The stored sections were processed at different timepoints (n = 11), along with sections freshly prepared from FFPE blocks (controls). A total of 297 sections (triplicates of rat brain, kidney and liver stored at either RT, -80°C or freshly prepared) were tryptically digested then analysed on Triple TOF 6600 mass spectrometers (SCIEX) in data-dependent acquisition (DDA) mode. Selected kidney digests were also analysed in data-independent acquisition (DIA) mode.
Results: ProteinPilot searches of DDA runs showed that the number of proteins and peptides identified and some common post-translational modifications (PTMs) were unaffected by the storage time or temperature. Nine of the PTMs specific for FFPE samples were then monitored for quantitative changes using the more reproducible DIA data and all were again unaffected by the storage time or temperature.
Conclusions: These results demonstrate that FFPE tissue sections are robust and suitable for proteomic studies for at least 1 year from the time of sectioning when fresh frozen tissues are not available.

P07.16

Identification of Key Protein Markers of Colorectal Cancer for the Development of the Disease by TMT-Quantitative Proteomics

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Introduction and objective

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death worldwide. A better understanding of the biology of CRC would help identifying specific protein markers of the disease that could be used as prognostic and/or diagnostic biomarkers or become potential targets of intervention. Here, we aim to analyze the differential protein expression in paired healthy and CRC tissues by quantitative proteomics to identify key proteins involved in the pathogenesis of the disease.

Methods

Tandem mass tag (TMT) experiments were performed using paired paraffin-embedded tissue samples of adenoma, adenocarcinoma and healthy tissues from 6 CRC patients. Proteins were identified by mass spectrometry using a Q-Exactive, and the subsequent data analysis was performed using MaxQuant and Perseus to identify proteins differentially expressed in CRC.

Result and discussion

More than 3000 proteins were identified and quantified from the TMT experiments. After data analysis, 156 and 150 proteins were observed as upregulated or downregulated, respectively, in adenoma and/or adenocarcinoma. After bioinformatics analysis, 12 altered proteins were selected to study their role in CRC by orthogonal techniques, using tissue and serum samples from patients and controls. Furthermore, loss-of-function assays with two isogenic CRC cell models and siRNAs against two candidate proteins allowed determining their association to the disease.

Conclusions

TMT experiments allowed the identification of proteins altered in CRC patients. The dysregulation of 12 of these proteins in patients was confirmed at mRNA and protein level by different techniques, being several candidate proteins altered in the sera of CRC patients, suggesting a key role of these proteins in the development of CRC or as biomarkers of the disease. In addition, two candidate proteins were found to disrupt the tumorigenic properties of CRC cells, indicating an important role of these proteins in CRC pathology.

P07.17

Automated Proteomics Sample Preparation of Extracellular Vesicles from Human Plasma and Serum

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Introduction: Extracellular vesicles (EVs) are ubiquitously secreted by almost every cell type and present in all body fluids. The blood-derived EVs can be used as a promising source for biomarker monitoring in disease. Current development in EVs proteomics have analyzed in clinical subjects. To date, researcher have developed the EV isolation methods, including differential centrifugation, sucrose gradient ultracentrifugation, size exclusion chromatography, affinity capture and asymmetric-flow field-flow fractionation. However, their isolation methods are limited in throughput for human subjects. Here, we introduced a novel automated EV isolation and sample preparation method for EV proteomics analysis that can be started with low volume of multiple clinical samples.
Methods: EVs were automatically separated from both EDTA plasma and serum of six healthy subjects (n=3) by an affinity capture isolation method using combination of Hamilton and Presto systems, and we applied them in Mass spectrometry, data-independent acquisition. In addition, the sample preparation for EV proteomics performed using combination single-pot, solid-phase-enhanced sample-preparation (SP3) technology with Flex system in 96 well format.
Results: Nanoparticle tracking analysis, Transmission electron microscopy and Western blot results identified EV population containing Microvesicles and exosome isolated from plasma and serum, and 4079 proteins were identified in total. Proteins related to complement and coagulation cascades and cholesterol metabolism were enriched in plasma EVs, and platelet activation were enriched in serum EVs. The protein profiling provided a catalogue of the differences in plasma and serum EVs between individual, and successfully showed the proteins as a reference for biomarker discovery.
Conclusions: We have successfully isolated EVs from blood using an automated isolation method and developed an automated method for EV proteomic sample preparation. This method is attractive for processing large sample batches and limited samples for biomarker development.

P07.19

Proteomic Profiles of Zika Virus-Infected Placentas Bearing Foetuses with Microcephaly

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Introduction: The possible molecular event that causes Congenital Zika Syndrome (CZS) in newborns must begin with the transplacental transmission of the virus from the mother to the foetus. This work aims to study the role of the placenta in ZIKV-induced microcephaly and understand the mechanisms of ZIKV to cross the placental barrier using a label-free quantification proteomic approach.
Methods: Three groups of placentas were studied: an uninfected control group (Ctr), a group of ZIKV infected placentas with normal neonates (Z+), and a group of ZIKV-positive placentas with microcephalic foetuses (MC+). After extraction with 7 M urea/ 2 M thiourea/ 2% SDC; 60 µg of proteins were reduced and alkylated with DTT and IAA respectively and digested with trypsin (1/25, w/w) for 16h at 37oC. Peptides were analysed by nLC-MSMS and protein identification and label-free quantification was achieved in Proteome Discoverer v2.4. Altered metabolic routes and biological processes were studied in the DAVID Bioinformatic Resources, the STRING, and the Reactome Pathway.
Results: Zika virus infection alters protein expression related to DNA damage and mRNA translation in the placenta. Viral transcytosis-related processes that could be associated with a possible vertical transmission route were also detected in ZIKV-infected placentas. The analysis of the MC+ vs Z+ group shows that most dysregulated processes in the MC+ group were related to cellular adhesion, suggesting an invasion of extravillous trophoblasts from the placenta towards the maternal decidua. We also detect dysregulation of proteins related to immune response indicating a disruption of maternal tolerance towards the foetus, that could trigger morphological malformations in the foetus brain
Conclusions: Placentas infected with ZIKV increased expression proteins related to transcytosis suggesting a probable route for vertical transmission. Increased expression of cell adhesion proteins and altered immune response may indicate disruption of maternal tolerance causing neurological malformations in newborns.

P07.20

Discovery of Prostate Cancer Biomarkers by Immunoaffinity Proteomics.

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Introduction: Prostate cancer is the most common malignancy in men. Prostate-specific antigen (PSA) is currently used for prostate cancer diagnostics, but has limitations, including low diagnostic specificity. Our aim is to develop immunoaffinity-mass spectrometry (IA-MS) assays for previously unstudied prostate-specific proteins and evaluate them as potentially novel biomarkers of prostate cancer.
Methods: LNCaP and VCaP prostate cancer cell lines were grown in RPMI-1640 and DMEM, respectively. RT-PCR was used to confirm gene expression. In-house time-resolved fluorescence ELISA was developed for relaxin-1 (RLN1) and TMPRSS2-ERG fusion proteins. Nanoflow reverse-phase chromatography and nano-electrospray mass spectrometry were used for protein quantification.
Results: Literature searches revealed that RLN1 (presumably a secreted prostate-specific protein) has never been validated in the context of prostate cancer or detected at the protein level. Using RT-PCR, we confirmed the expression of RLN1 and TMPRSS2-ERG transcripts in LNCaP and VCaP cells, respectively. To investigate expression of endogenous RLN1, we developed and optimized in-house ELISA (LOD 60 pg/ml) and IA-MS assays (LOD 300 pg/ml). Endogenous RLN1 was detected in some seminal plasma and serum samples but not in the LNCaP cell line secretome. To investigate expression of TMPRSS2-ERG fusion protein, we developed and optimized in-house ELISA (LOD 60 pg/ml) and IA-MS assays (LOD 0.39 fmoles on columns). Low-abundance TMPRSS2-ERG protein was quantified in VCaP cells and FFPE prostate cancer tissues. Results from both assays were in good agreement (1).
Conclusions: IA-MS assays provide orthogonal tools to measure low-abundance prostate-specific proteins and evaluate them as potential biomarkers of prostate cancer.

1. Fu, Z., et al. 2021 Mol Cell Proteomics 20; 1535-9476

P07.21

A Multi-Omics LC-MS Approach for Rational Selection of Neo-Antigens and Unbiased Detection of Corresponding Neo-Epitopes from Low Number of Cells

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In the context of anti-tumour immunotherapy and vaccine design, mass spectrometry (MS) is a powerful technology for the detection of neo-epitopes presented on the surface of tumour cells by the human leukocyte antigens (HLA). MS can be used mainly in two different ways for the identification of epitopes, which have their distinct strengths and weaknesses. The common "data-dependant acquisition" allows the unbiased identification of HLA-presented peptides; however, it is limited by its low sensitivity. The targeted-MS approaches are more sensitive, however biased by the HLA binding prediction algorithms in addition to the fact that a rigorous prioritization is needed to reduce the large list of predicted neo-epitopes to a manageable number of peptides. In order to increase the success rate of neo-epitope identification per sample and the sensitivity of detection, we devised a 2-step MS methodology in combination with DNA and RNA sequencing. The first step consists in the analysis of an HLA-peptide immuno-precipitation in an untargeted manner to identify any peptides derived from neo-antigens using a patient-specific protein sequence database. This step, in addition to allowing for the potential identification of neo-epitopes of higher abundance, is introduced especially to identify mutated proteins that undergo proteasome degradation and HLA presentation on the surface of tumour cells. Candidates with sufficient mutated allele expression (mRNA sequencing) are then selected for in silico epitope prediction. The second targeted-MS step specifically looks for predicted neo-epitopes with higher sensitivity, potentially allowing for the identification of lower abundance neo-epitopes.
So far, the lowest number of cells used for the detection of tumour-derived neo-epitope was 50 million cells known for their high mutational load. Deploying our multi-omics approach on a patient-derived pancreatic cancer xenograft cell line, we were able to identify three candidate neo-epitopes, two of which can be detected from as low as 2.5 million cells.

P07.22

Label-free Proteomics Profile from Spleens of Lupus-like cGVHD WT Mice Reflects a STAT-1-driven Type I IFN-signature

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Introduction: It was of interest to test whether the augmented serum levels of IL-27, and increased T-bet expression found in B cells from cGVHD WT mice and not in CD38-deficient mice had a proteomic profile sustaining these findings.
Methods: Protein extracts were analyzed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Amazon Speed, Bruker) at IPBLN-CSIC Proteomic Facility. Protein identification was done with ProteinScape 4.0 (Bruker) and MASCOT 2.4 data searching using the SwissProt database. For label-free proteomic quantification we used the exponentially modified protein abundance index (emPAI) implemented into the MASCOT data searching platform. Two biological samples per mouse type and 3 technical replicates per biological sample were analyzed (Proteomic Data are available via ProteomeXchange with identifier PXD026947). We used ClueGO_v2.5.8 and CluePedia_v1.5.8 within the Cytoscape_v3.8.2 software environment for functional enrichment analysis of the lists of identified proteins. Results are visualized as networks in which Gene Ontology (GO) terms and pathways are grouped based on their biological role. CluePedia allows to expand ClueGO terms into nested networks with associated genes.
Results: Volcano plots showed significant differences in protein abundance in the spleen lysates from bm12>Cd38-/- mice versus bm12>WT mice. Among the proteins which showed increased abundance in spleens of bm12>WT mice vs bm12> Cd38-/- was STAT1. ClueGO functional enrichment analysis showed STAT1 associated with a cluster of proteins in GO terms including positive regulation of type I IFN production, type I interferon production, positive regulation of interferon-alpha production, interferon-beta production, and cellular response to IL-7. Positive regulation of type I interferon production was investigated in a subnetwork using the CluePedia plug-in. STAT1 was clearly involved in the regulation of the type I IFN signaling pathway with other identified proteins.
Conclusions: Our data suggest that Label-free proteomics is useful to dissect signaling pathways associated with lupus disease.

P07.23

Cardiac Sex Disparities are Established Prior to Gonad Formation

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Introduction:
Human sexual dimorphism is associated with differences in the prevalence of various disease states. Sex disparities exist in the anatomy and physiology of cardiac tissues and in the preponderance of specific types of heart disease. Clinical studies have implicated sex hormones as an influencing factor in differing patient outcomes. However, current studies suggest that a sex-specific program controlled genetically through the sex chromosomes outside of the sex organs to control cardiac protein expression. Although hormones are critical in cardiac disease, the mechanisms underlying sex differences in cardiac homeostasis and disease remain unexplained.

Methods:
RNA-seq and tandem mass tags mass spectrometry (TMT MS) were used to identify transcripts, proteins, and pathways differential in the Collaborative Cross (CC) model, a surrogate for human diversity. To probe whether sex disparities in cardiac protein expression result from sex chromosome or hormone mechanisms, we quantified proteins in adult cardiac tissue derived from the Four Core Genotypes (FCG) model. The role of X-linked gene dosage was next examined by TMT MS using Turners syndrome (XO) and Klinefelter (XXY) mouse models. Finally, to determine when, during embryogenesis, heart tissue displays sex disparities in protein expression, we analyzed hearts at E9.5.

Results:
We identify processes diverging between males and females across heterogeneous populations. Contrary to current dogma, cardiac sex disparities are not only controlled by sex hormones, but also through a sex chromosome mechanism, which is established by X-linked gene dosage. Additionally, cardiac sex dipartites occur at the earliest stages of heart formation, preceding gonad formation. Finally, we establish a role for A1BG in cardiac defects in females but not males.

Conclusions:
Our findings imply that cardiac sex differences are initiated by X-linked genes that act via a dosage-specific mechanism early in development. Our study provides new insights into sex-biased cardiac disease and developing sex-specific therapeutic interventions.

P07.24

Serological Profiling of Crohn's Disease and Ulcerative Colitis Sera Reveal Microbial Antibody Markers

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Introduction
Inflammatory Bowel Disease (IBD) represents a group of intestinal disorder with two different clinical phenotypes: Crohn's disease (CD) and ulcerative colitis (UC). The public health burden of IBD is rising globally. Current methodology to detect the onset of the disease is a combination of histopathology and endoscopy. There is a need for non-invasive serological markers to reveal the disease state. The intestinal microbiota plays an important role in IBD pathogenesis. People developing IBD are found to have a disbalance between commensal and pathogenic bacteria which is referred to as dysbiosis.

Methods
We used a protein microarray displaying 1,572 microbial antigens from 48 different bacteria and 33 different viruses. The microarrays were probed with 100 CD, 100 UC and 100 healthy subjects' sera. The antibodies from the sera bound to the microbial antigens were detected with a secondary antibody having a fluorophore. The microarrays were scanned in a laser scanner to determine the fluorescence intensity of each antigen. ArrayPro software was used to extract the raw data. The values obtained were median normalized to minimize the microarray-to-microarray variation. These data were used for further statistical analysis.

Results
We identified several novel flagellins and four non-flagellins markers (antibodies) elevated in CD compared to healthy controls. We found that antibody response decreases in UC patients compared to healthy, which can be due to dysbiosis. We also found that species like B. vulgatus and C. koseri have potential role in IBD progression. When the autoantibody response to same set of antigens were compared to microbial antibodies, there was no correlation for their presence in sera. The humoral immunity is strongly activated in CD compared to UC, with relatively a smaller number of microbial antibodies in UC.

Conclusions
We discovered some novel biomarkers for the early detection and management of IBD.

P07.25

Quantitative Proteome Profiling ties the Complement System to Amyloidosis

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-Introduction
Amyloidosis is a disease group caused by aggregation of insoluble, fibrillar protein in diverse tissues. In addition to the major fibril protein, amyloid deposits also contain non-fibrillar constituents which may be involved in pathophysiology. The role of these additional factors in disease progression gained increasing interest in recent years, shifting the focus of research from purely diagnostic to exploratory. Consequently, proteomics has become a valuable tool for diagnostic typing of amyloid deposits as well as biomarker discovery. The analysis of amyloid tissue from patients is most frequently performed by accurate laser microdissection of amyloid deposits followed by discovery-based bottom-up LC-MS to generate qualitative datasets, with the most evident protein identifications usually defined as amyloidogenic and amyloid-associated.

-Methods
We employed an alternative approach and analyzed tissue sections containing amyloid deposits by quantitative mass spectrometry-based proteomics. Following manual dissection, tissue samples of equal size and with heterogeneous amyloid load were dissected and forwarded to bottom-up proteome analysis and label-free protein profiling. Amyloid-associated proteins were identified by a correlation-based approach.

-Results
The amyloid protein was identified in all samples, with full sequence coverage and with a plethora of modifications. Using the major amyloid protein as bait, correlating LFI profiles were identified among the dataset. By this method, amyloid-associated proteins could be confidently differentiated from the normal tissue matrix because they showed significant correlations of label-free intensity profiles.

-Conclusions
A comprehensive list of proteins spatially enriched in amyloid deposits was discovered which showed clear functional association to the disease. In addition to well-known signature proteins (e.g., apolipoprotein E, apolipoprotein A-IV, and vitronectin), most of the members of the complement system, including all seven components of the membrane attack complex could be linked to the disease. These data support the hypothesis that the complement system is activated in amyloidosis.

P07.26

Clinical Mass Spectrometry Center Munich (CLINSPECT-M): Adding a Proteomic Component to Molecular Tumor Boards

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Introduction
Gliomas are one of the most common types of brain tumors with very limited treatment options and poor patient survival rates. Over the last decade, progress was made in the classification of gliomas and genomic analysis revealed some key driver genes. However, druggable targets and biomarkers which may allow personalized therapy are still missing. Therefore, we analyzed the proteome of 400 retrospective adult glioma samples from the biobank of the TU Munich in order to elucidate their proteomic profile and search for new targetable finger prints.

Methods
Aiming for deep proteome coverage, Eckert and Chang et al. optimized a proteomic workflow for FFPE material. High efficient protein extraction and de-crosslinking was achieved by boiling the tissue in 2% SDS, Tris buffer (500 mM, pH 9). Proteins were digested using the SP3 approach on an automated liquid handling platform ensuring high reproducibility. Peptides were loaded onto EvoTips followed by LC-FAIMS-MS/MS measurement using 2x 88 min gradients (5 CVs, 600 ng digest each) on an Exploris 480. Data processing was performed with MaxQuant and Protrider, a tool based on denoising autoencoders that allows pinpointing driver proteins within a patient dataset without the need of a control group.

Results
In order to limit the proteomic analysis to the tumor itself while excluding surrounding tissue e.g. necrotic areas, each tissue slice was pathologically classified. The area of interest was collected manually and this relatively pure tumor material was analyzed using the FFPE workflow above. Overall, we profiled 400 glioma samples covering >4,000 protein groups per sample. Bioinformatic analysis uncovered profiles distinguishing subtypes and highlighted candidates of oncogenic driver proteins.

Conclusions
Using gliomas as an example, the framework of the CLINSPECT-M provides the foundation for systematic integration of proteome profiling into molecular tumor boards and personalized therapy.

P07.27

Peptide and Metabolite Profiling in Histological Variants of Papillary Thyroid Carcinoma

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Introduction: The characterization and diagnosis of follicular variant papillary thyroid carcinoma (FV-PTC); encapsulated (eFV-PTC), invasive (iFV-PTC) can be challenging in pathology. Due to the indolent nature of noninvasive eFV-PTC, the nomenclature was revised and termed as; noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP). Earlier, we showed differentiation of NIFTP from normal thyroid paranchyma (1). In this study, we aimed to evaluate the use of matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI) to further characterize eFV-PTC, iFV-PTC, and NIFTP.
Methods: FFPE tissue samples were sectioned at 3 µm thickness. The slides were washed with xylene and then coated with 10 mg/ml 9-Aminoacridine (9-AA) in 70% methanol for metabolite analysis. Mass spectra were acquired in negative ion mode (m/z 50-1000) using Rapiflex MALDI Tissue Typer (Bruker Daltonics GmbH, Bremen, Germany). For peptide analysis, previously established protocol was used (1).
Results: Each slide contained 20 very small tissue cores including e-FV-PTC, i-FV-PTC, and NIFTP. Unsupervised hierarchical clustering analysis revealed certain peptides and metabolites that can discriminate different histological variants of PTC. Additionally, network analysis showed that certain tissue cores in each group had similar peptide and metabolite profiles.
Conclusions: High-throughput MALDI-MSI revealed practical metabolite and peptide information for the characterization of different histopathological variants of PTC.

1. Ucal et al. 2019 Thyroid 29(8):1125-1137.

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P07.28

Serum Proteomic Analysis of Severe Eosinophilic Asthma Patients before and after Two New Biological Therapies

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Introduction: Severe eosinophilic asthma (SEA) is typically characterized by chronic airway inflammation, oxidative stress and elevated proinflammatory cytokines, especially IL-5. Mepolizumab and benralizumab are both humanized IgG antibodies directed against IL-5 and IL-5 receptor, respectively, approved for SEA control. In order to clarify the follow-up of therapies and to identify biomarkers to establish the optimal treatment duration, persistence of effectiveness and safety, we had previously conducted a proteomic analysis of SEA serum samples before and after 1 month of both treatments, with interesting results. Here we performed a further differential proteomic analysis introducing also SEA sera after 6 months of both therapies and sera from healthy patients.
Methods: Differential proteomic analysis was performed comparing SEA patients' sera before monoclonals treatment (T0) and after one (T1M) and six months (T6M) of mepolizumab and benralizumab (T1B-T6B) therapies and healthy control (CTRL). Identified proteins were used to perform enrichment analysis by MetaCore software. Proteins of interest were validated by immunoblot.
Results: Differential proteomic analysis highlighted 82 differences among the six conditions. T-SNE and heatmap analysis showed that T0 and T1 samples were influenced by the different treatments, on the contrary all T6 samples converged to CTRL samples regardless of treatments. In view of the bioinformatic results, we validated differential proteins abundance by immunoblotting analysis, obtaining an increased level of ceruloplasmin already after one month of benralizumab administration; while in mepolizumab the ceruloplasmin increment was visible only after six months of therapy. Also, we detected up-regulation of plasminogen after both treatments, positively correlated with timepoits, and a dysregulation of ApoA1 different isoforms.
Conclusions: By proteomic approach, we identified several protein species which changed in abundance during the two treatments follow-up, highlighting the general restoring trend of T6 proteomic profiling to that of the control. Further analysis is needed to investigate about potential altered pathways.

P07.29

Mass Spectrometry-Based Proteomic and Metabolomic Profiling of Serum Samples for Discovery and Validation of TB Diagnostic Biomarker

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INTRODUCTION: Tuberculosis (TB) is a transmissible disease listed as one of the 10 leading causes of death worldwide (10 million infected in 2019). A swift and precise diagnosis is essential to forestall its transmission, for which is crucial the discovery of effective diagnostic biomarkers. METHODS: Two independent cohorts comprising 22 and 28 subjects were assayed by proteomics. While 44 subjects were included for metabolomic analysis. All subjects were arranged into 2 experimental groups – healthy (H) and patients (P). Proteomics workflow comprised the tryptic digestion of the depleted serum. LC-MS/MS protein and metabolite levels were submitted to multivariate, univariate and ROC analysis. An integrated ROC analysis was also performed for the 36 common individuals in the proteomic and metabolomic sets. RESULTS: From the 149 and 79 proteins identified in each set, four were found to be differentially abundant in both cohorts ($p>0.05$; FC>±1.5). The AUC, specificity and sensitivity determine by ROC statistical analysis for each proteomic set were 0.96; 86% and 100%; and 0.99; 100% and 85%. PLS-DA models created with the metabolites quantified in both modes: 69 (positive mode) and 32 (negative mode) allowed the discrimination between H and P. AUCs determined by ROC analysis comprising 5 metabolites for each mode were above 0.99 with all samples being correctly assigned to the respective experimental group. The determined parameters for the integrated ROC analysis enrolling the 14 elected biomarkers (AUC=1, specificity=100% and sensitivity=100%) and has correctly assigned the 8 individuals used only for prediction. CONCLUSION: This multi-omics approach suggests 4 proteins and 10 metabolites as potential biomarkers for tuberculosis diagnosis. Two of the proteins are involved in antibacterial immune response. Validation of the proposed biomarkers require target analysis with a bigger cohort.

P07.30

Cell Type Deconvolution of Brain Proteomes (BrainDecon)

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Introduction: Small-sample and bulk proteomics studies of the brain aim to uncover proteins that mediate phenotypic differences between samples. However, unlike for RNA-seq data, there are no readily accessible methods for inferring cellular composition within individual samples. Given the well-annotated differences in cell type composition among anatomical regions of the brain and the role played by specific cell types in neuropathologic conditions including cancer, this is an important source of phenotypic variability. Thus, a limitation of current proteomic studies is the inability to distinguish whether observed differences in protein abundance between samples are caused by shifts in the cellular composition, altered protein expression, or both.

Methods: To meet this challenge, we leverage proteomes of flow-sorted cells from major cell types of the adult mouse brain, including neurons, astrocytes, oligodendrocytes, and microglia. Using non-negative least squares (NNLS) regression, we fit cell-type-specific protein expression profiles to target datasets to infer the relative contributions of each cell type to the overall signal. Analysis of residuals enables identification of sample-specific and protein-specific uncertainties.

Results: We validate our method using mouse and human datasets from brain regions where the cell type composition is known. By using mouse-derived cell-type-specific profiles for both mouse and human data, we also assess the utility of our approach to cross-species analysis. Finally, we evaluate the accuracy of the method using samples with matched RNA-seq and TMT proteomics data generated from the same cells, to benchmark our results from proteomics data against best-in-class methods for cell type deconvolution from RNA-seq.

Conclusions: Using high-quality cell-type-specific reference proteomes, we propose an NNLS approach to deconvolution of major cell types of the brain. The methodology can be applied to a variety of global proteomics datasets from human and mouse brain and will enhance our ability to interpret proteomic variation in health and disease.

P08.01

Development of a Targeted Proteomics Method for Serum IGF-I, IGF-II, IGFALS and IGFBP-1, 2, 3, 4, 5 and 6.

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Introduction:
Measurement of serum or plasma Insulin-like Growth Factor I (IGF-I) and IGF Binding Protein 3 (IGFBP-3) is used in the diagnosis of several diseases, including Growth Hormone (GH) deficiency (associated with dwarfism and short stature) and GH excess (associated with pituitary gigantism and acromegaly). IGF-I is also used in the monitoring of GH therapy, and has been suggested as a marker to detect GH abuse among professional athletes. In addition, several other members of the IGF family of proteins are of clinical interest. Targeted proteomics is compatible with MS-platforms already implemented in clinical laboratories (i.e. uHPLC coupled to triple-quadrupole MS) and allows for multiplexing. Here we aim to develop a targeted proteomics method for simoutaneous quantification of serum concentrations of IGF-I, IGF-II, IGFBP-1, -2, -3, -4, -5 and -6, and IGF Acid Labile Aubunit (IGFALS).
Results:
Through a process of testing and optimization a robust, sensitive and practical method of serum preparation and LC-MS/MS analysis was identified. Optimal tryptic peptides and transitions for IGF proteins were identified by combining experiments with database searching. Suitable internal standards were either synthesized (stable-isotope labeled (SIL) tryptic peptides) or purchased (full length recombinant SIL-IGF-I). Optimal calibrant solutions were compared by spiking recombinant IGF proteins into different test matrices. Quality control samples were generated by spiking recombinant IGF proteins in pooled human serum.
Conclusions:
The method appears promising for multiplex measurement of IGF proteins in serum and the work-flow is compatible with a clinical routine setting. Method validation according to CLSI guidelines is presently ongoing.

P08.03

Bench-to-Bedside Alzheimer Disease's Detection by Biosensing Approaches Detecting Autoantibody Biomarkers Identified by Protein Microarrays-Based Proteomics

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Introduction: Alzheimer's disease (AD) is a progressive and chronic neurodegenerative disorder. It is the most common form of dementia worldwide, with a 10-30% prevalence in the ageing population (>65 years of age). In AD brain, drastic changes take place, including the alteration of the blood-brain-barrier and the permanent inflammatory state of the brain. These may lead to the generation of AD-specific autoantibodies that could be used for the detection of the pathology. Thus, we here aimed to identify and validate AD-specific autoantibodies and their target proteins as biomarkers of the disease.
Methods: Protein microarrays-based proteomics were used for AD-specific autoantibody targets identification. Validation of identified proteomic targets together with frameshift-aberrant APP+1 and UBB+1 peptides was performed by luminescence in-solution immunoassays. Purified to homogeneity HaloTag fusion peptides as bioreceptors were used for the construction of amperometric biosensing platforms with the aim to get a POCT-like device for AD detection.
Results: For autoantibody identification, two T7 phage display libraries displaying the cDNA repertoire of AD patients and healthy individuals' brain were biopanned and 1920 unique phages and controls were printed on nitrocellulose microarrays. After protein microarray screening with serum from AD patients and controls, we identified four peptides target of autoantibodies as potential biomarkers of the disease. By luminescence in-solution immunoassays, these peptides together with APP+1 and UBB+1 frameshifts peptides showed AD diagnostic ability. Then, purified peptides expressed as HaloTag fusion proteins were used to construct the first sensing bioplatform based on the use of this type of receptors for AD detection. After optimization of key variables, the amperometric biosensing platform analytical operational characteristics demonstrated a highly significant clinical diagnostic potential.
Conclusions: Our results suggest the possibility of reliably and minimally invasively diagnose AD by using amperometric biosensing platforms detecting autoantibodies against AD-specific targets identified by proteomics.

P08.04

Precision Analysis Reveals Diagnostic Protein Biomarkers of Japanese Encephalitis Virus Infection in Cerebrospinal Fluid

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Introduction:Japanese encephalitis virus infection (JE) is a leading neurological infection in humans in Asia, and a significant public health problem. The standard diagnostic, anti-JEV IgM capture ELISA, has poor sensitivity (50-80%) in field testing, and poor specificity relating to cross-reactivity or previous infection with co-endemic flaviviruses, or vaccination. No rapid diagnostic is currently suitable for clinical use and insufficient laboratory capacity means that estimation of JE epidemiology or effectiveness of vaccination are major challenges. We sought to identify protein biomarkers in cerebrospinal fluid (CSF) to inform development of novel diagnostics.

Methods:We performed a pilot (n=15) followed by a larger study (n=148) of JE cases confirmed by gold standard seroneutralisation vs. controls of other confirmed neurological infections. CSF was analysed using untargeted liquid chromatography tandem mass spectrometry (LC-MS/MS); multiple batches of samples labelled with tandem mass tags (TMT), offline high pH reverse phase fractionation (100 fractions concatenated into 44 for deep CSF proteome coverage) and subsequent low pH reverse phase UHPLC with a Dionex Ultimate 3000 nano coupled to Q Exactive benchtop hybrid quadrupole-Orbitrap MS. Data was processed using Proteome Discoverer 2.5 (Sequest and Percolator) with statistical analyses performed in Rv4.1.0 (MSstatsTMT package).

Results:4,630 proteins were identified consisting of 4,181 human and 449 pathogen (bacterial or parasitic) proteins. 4,092 human proteins were quantified, of which 1,770 were quantified in all samples. 271 human proteins were detected at differential levels in JE cases vs. controls. 14 human proteins were identified in all JE samples and no controls.

Conclusions:This is one of the largest studies of CSF in patients with neurological infections investigated by untargeted LC-MS/MS resulting in a large and diverse set of proteins. Proteins differentially-expressed in JE cases informs understanding of the host response with potential for identifying therapeutic targets. Subsets of proteins have been chosen for validation through antibody-based methods.

P08.05

Application of 16O/18O Labeling in Characterization of Thyroid Cancer Patient

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Introduction
Stable isotope labeling was widely introduced to proteomic study. Among these, 16O/18O labeling is catalyzed by trypsin digestion. For clinical analysis of thyroid cancer, however, the quantitative analysis by mass spectrometry was little studied. We introduced 16O/18O labeling as a strategy to study thyroid cancer in the present study.

Methods
Soluble proteins isolated from cancer tissues and normal tissues around (control) of a patient with thyroid cancer were labeled by 16O and 18O individually through trypsin digestion. After combination of the two samples, the sample was subjected to mass spectrometry analysis using quadruple time-of-flight (Q-TOF) for protein identification. The 16O/18O ratio was calculated by at least two quantification softwares.

Results
The marker protein thyroglobulin was successfully labeled by 16O/18O. More than two unique peptides were identified with quantitative ration. The results showed ratios of peptides from cancer tissues with significantly different ratios from the control.

Conclusion
Our results provide quantitative information on the peptide level. With this approach, one can apply to clinical study to trace marker protein in patients with thyroid cancer.

P08.06

Tumor Outflow Pulmonary Blood Derived Exosome GCC2 act as a Clinically Informative Biomarker in Patients with Surgically Resected Lung Adenocarcinoma

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Introduction: Lung cancer is one of the most diagnosed cancers and the leading cause of cancer associated deaths. Exosomes are nano-sized vesicles that are secreted by all type of cells and involved in biological functions. Accumulating evidence supports a role for exosomal protein in diagnosis. Tumor draining pulmonary vein blood (TDPV) is more abundant in cancer associated molecules than peripheral blood. We previously demonstrated that TDVP derived exosomes were increased than periphery and exosomal GCC2, which was identified by exosome proteomics, act as potential lung adenocarcinoma biomarker with cancer progression. The purpose of this research is to evaluate the quantification of exosome GCC2 in TDPV versus periphery of animal cancer model and lung cancer patients who received surgery as a potential biomarker for precise cancer diagnosis.
Methods: Rabbit animal model and human subjects were used in this study. Blood sample was collected via the peripheral vein from all groups, and pulmonary blood was collected intraoperatively from all groups, except the healthy group. Blood plasma derived exosomes were isolated by size exclusion chromatography and analyzed by nanoparticle tracking assay, western blot, immunogold label TEM, GCC2 enzyme-linked immunosorbent assay.
Results: The lung cancer animals and patients show that the increased level of exosome GCC2 compared with healthy animals and human subjects. The level of exosome GCC2 isolated from TDPV revealed that animal lung cancer animal model and patients increased levels than peripheral blood . The increasing trend of exosome GCC2 in TDPV showed higher correlation with pathological stages of lung cancer patients than that of the periphery . Through the statistical analysis, TDPV exosome GCC2 could provide meaningful clinical information to patients with underwent lung cancer surgery than peripheral exosome GCC2.
Conclusion: Exosome GCC2 in TDPV is a promising and clinically informative biomarker for lung cancer patients who received surgery.

P08.07

Multi-Staged Enrichment Method Capable of Quantifying Mutant Frameshift MUC1 in Urine from Patients with Autosomal Dominant Tubulointerstitial Kidney Disease (ADTKD-MUC1)

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Introduction
ADTKD-MUC1 is a rare kidney disease caused by a frameshift mutation in Mucin1 that results in a truncated neoprotein (MUC1-fs), leading to toxic proteinopathy. Genetic diagnosis of ADTKD-MUC1 involves a massively parallel sequencing that is not widely available. We developed a two-step enrichment method using LC-MRM-MS (EV-iMRM) capable of detecting and measuring MUC1-fs in ADTKD-MUC1 patient urine.

Methods
Four peptides (two unique to MUC1-fs, two unique to wild-type MUC1) were used to generate purified anti-peptide polyclonal antibodies (Abs). Stable isotope-labeled (SIL) peptides were synthesized for use as internal standards.

Extracellular vesicles (EVs) were extracted from urine using a bead slurry. EV lysates were digested with trypsin, spiked with an equimolar mixture of SIL peptides and enriched using a plex of Abs crosslinked to Protein-G beads. Captured peptides were eluted using a KingFisher magnetic bead processor. Eluates were desalted on the AssayMAP Bravo robot and analyzed by LC-MRM-MS.

Results
The performance of each peptide in the multiplexed iMRM assay (LOD, LLOQ, linear range and reproducibility) was characterized using a reverse response curve prepared in EVs enriched from control urine. The linear range was determined to be 0.025 to 18 fmol/µg with an overall median CV threshold of <15%.

MUC1/MUC1-fs was quantified in ADTKD-MUC1 patient urine (n=44) from Wake Forest University and University of Cyprus and Receiver Operating Characteristic (ROC) curves were generated. For MUC1-fs, we determined one peptide to have 95% specificity and 91% sensitivity (AUC = 0.96). MUC1 wild-type peptides were specific (95%), but not sensitive (20%) for ADTKD-MUC1 samples (AUC = 0.63).

Monoclonal antibodies with comparable enrichment efficiency and specificity were then generated for clinical applications.

Conclusions
We developed a sensitive, two-stage enrichment, high-throughput iMRM assay for clinical trial applications, the first of its kind to quantify the MUC1-fs neoprotein in ADTKD-MUC1 patient urine.

P08.08

Identification of Procalcitonin in Septic Patients Serum by Affinity Chips and Mass Spectrometry

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Introduction:
Sepsis is a worldwide health condition caused by a disproportionately large immunity system response for pathogen presence. Fast diagnosis can make a difference in the survival of the patient. For the purpose of clinical diagnosis of sepsis, a serum protein called procalcitonin (PCT) is often used. Procalcitonin concentration in the bloodstream correlates with sepsis severity and increases up to a thousand times in a short period. This study was aimed to develop a MALDI MS-compatible method for in-situ enrichment of PCT from patient's serum. The objective of this study was in-situ enrichment and MS characterization of PCT forms from septic patients sera.

Methods:
The MALDI-compatible immunoaffinity chips were prepared by surface modification of indium-tin-oxide coated glass slides (ITO) by ambient ion soft landing using an anti-PCT antibody. The chips were used for in-situ enrichment of PCT from serum treated by acetonitrile. After incubation, chips were washed, and spots were covered by a MALDI matrix. The enriched PCT was measured by MALDI TOF (Bruker Daltonics) in linear positive mode.

Results:
Recombinant PCT was used for the optimization of the procedure. Acetonitrile precipitation of human serum increased the sensitivity of the method dramatically. The optimized method used to monitor PCT in human serum reaches the limit of detection 10 ng/mL. The native human PCT was observed at m/z 6306 (2+) and m/z 12620 (1+). The high-resolution mass spectrometry using MALDI-FTICR uncovered different forms of PCT in patient samples.

Conclusion:
The functionalized MALDI surfaces prepared by ion soft landing were successfully used for in-situ enrichment and detection of PCT from human serum, where its different forms were observed in patient's samples. The method is fast and robust and might be potentially used in clinical diagnostics.

P08.09

Proteomic Analysis of Synovial Liquid to Search for Severity Biomarkers in Osteoarthritis

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Introduction: Limitations on early diagnosis and effective pharmacotherapy for osteoarthritis (OA) are predominantly attributed to the current limited understanding of its pathogenesis. Pathological changes in the joint are expected to be represented by synovial fluid (SF) proteins, which are altered due to the disease and have significant biomarker potential. In this study, a proteomic analysis based on label-free quantification (LFQ) has been performed to explore the protein profiles of SF from different grades of OA and healthy controls.
Methods: Post-mortem SF samples (n=60) from knee joints were used. Joints were graded based on the severity of changes in the knee cartilage surfaces using the Outerbridge scoring system, which grades joints from grade 0 (n=5), GI (n=24), GII (n=20), GIII (n=7) and GIV (n= 4), Twenty µg of SF proteins were analyzed by LC/MS/MS on a nanoElute-LC coupled to a high-resolution TIMS-QTOF (timsTOF Pro, Bruker Daltonics).
Results: Proteins were quantified using the LFQ algorithm of MaxQuant software. The option of 'match between runs' was used for nonlinear retention time alignment. Further statistical and bioinformatic analyses were performed using MStats software. Around 1085 protein groups and 1427 proteins were identified in the SF samples, and over 90 protein groups were relatively quantified between the different grades of OA and healthy donors, many of them related with articular cartilage. Several proteins were increased in control samples compared to different OA grades, as collagen type XIV alpha 1, insulin like growth factor binding protein 5 or fibulin 1. In contrast, glutathione peroxidase 1 and glutathione S-transferase mu 1, among others, were increased in advanced OA compared to early stages.
Conclusions: Our study shows a distinct protein profile in synovial fluids from individuals with different OA grades and healthy donors, and reports potential clinically useful protein biomarkers for OA diagnosis and monitoring.

P08.10

An Intelligent Hybrid-Dia Data Acquisition Strategy for Cracking the Clinical Sample Complexity Challenge in Translational Proteotyping

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Introduction
MS-based proteotyping has been widely employed for biomarker discovery, yet the clinical/translational proteotyping community requires strategies that not only enable the discovery of novel biomarker candidates but can also boost the probability of establishing protein-based biomarker assays, enhance analytical and clinical validation speed, and resolve the issue of data missingness in a quantitative protein matrix. Here we present an intelligent data acquisition Hybrid-DIA strategy enabling the comprehensive digitization of a clinical specimen while at the same time enhancing measurement sensitivity for a specific set of markers of clinical interest.

Methods
The Hybrid-DIA strategy consists of a standard DIA scan cycle, where MS1 scans are followed by several DIA MS/MS scans. Fast (multiplexed) PRM-MS/MS scans are triggered based on the detection of isotope-labeled reference peptides and serve as a second layer of confirmation. Successful isotope-labeled peptide detection triggers the high-quality measurement of the corresponding endogenous counter-peptide, multiplexed (msx) with the isotope-labeled peptide through msxPRM acquired with narrower isolation window width and maximizing ion injection time for each species. This data acquisition scheme maximizes instrument productivity and, in turn, results in only minor decrease in DIA acquisition time.

Results
We tested Hybrid-DIA on a pool of 185 representative proteotypic peptides for tumor-associated antigens. We generated mixes containing both the heavy reference peptide as well as its synthetic light isotope. Whereas the heavy reference peptide was kept constant, its light counterpart was measured in a dilution series ranging from 100 femtomole to 100 atomole. Preliminary data show that for some of the peptides we monitored we observed a lower LOD/LOQ for msxPRM than for DIA, as well a a lower CVs at lower peptide concentrations.

Conclusions
We could show that Hybrid-DIA has the potential to monitor clinical marker peptides at a better sensitivity and specificity than DIA alone.

P08.11

Cyclic Ion Mobility-Enabled Mass Spectrometer and Application to High Throughput Plasma Proteomics

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Introduction
Prostate cancer is a leading cause of cancer deaths for men in the U.S and numerous OMIC-based studies into the disease have been conducted, proposing potential markers. However, in order to provide a comprehensive and statistically valid data set, samples from a large cohort of individuals are required. This ultimately provides an analytical challenge, particularly for proteomics research where nanoscale chromatography is routinely adopted. Here, we evaluate Cyclic IMS for high throughput proteomic profiling of plasma from prostate cancer individuals.

Methods
Pooled samples corresponding to different disease states or treatments were created from 520 prostate cancer patients. These plasma samples were subjected to reduction, alkylation and trypsin digestion. Plasma digest samples were separated using 2.1mm scale chromatography at a flow rate of 150uL/min with a turnaround time of 25 minutes. The liquid chromatography system was coupled to an IMS oa-QTof mass spectrometer and data were obtained using an ion mobility enabled DIA method, HDMSE. Data were processed using a variety of informatic tools and searched with respective databases.

Results
The acquired dataset was imported and processed using both ProteinLynx Global Server and Progenesis QI for Proteomics and searched against a Uniprot Homo sapien database limited to 1% FDR. The samples were then assigned to their pooled groups, revealing a significant number of proteins with differential regulation between the sample groups. Proteins occurring in a minimum of two of the biological replicates and with ANOVA $p < 0.05$ were considered as significant and peptides associated with these were further analysed where multivariate analysis showed clear separation between the different groups. Curated data was then subjected to pathway analysis in order to provide their biological significance.

Conclusions
Cyclic IMS has been evaluated for high throughput proteomic profiling of plasma from prostate cancer individuals with biological significance derived from pathway analysis.

P08.12

Species-Specific Cutaneous Protein Signatures of Incision Injury and Correlation with Distinct Pain-Related Phenotypes in Humans

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Introduction: Postsurgery pain and chronification processes are major clinical challenges. Due to incomplete knowledge of underlying mechanisms, treatment options and preventive measures are limited. To overcome the 'translational gulf' of bidirectional approaches, knowledge about differences and similarities between humans and rodents must be expanded. Additionally, experimental pain models in humans can help phenotyping of pain and identify mechanisms directly in humans. We performed an unbiased quantitative proteomic screen of differentially regulated proteins (DRPs) upon incision in human and mouse skin. Additionally, human volunteers were grouped based on their pain-related symptoms.

Methods: Skin biopsies from 26 human volunteers (ipsi,con) and 24 mice (incision and sham) were sampled using corresponding experimental incision models. Both study subjects received an unilateral incision (humans-volar arm, mice glabrous-hind paw). Human volunteers underwent psychophysics testing (questionnaires and quantitative sensory testing) pre- and post-incision. Grouping of volunteers was based on their hyperalgesic area (high and low responders). Skin biopsies 24h post-incision were analyzed with DIA-LC-MS. Species-specific and responder-specific DRPs were subjected to functional network analysis.

Results: Distinct overall changes of the skin proteome after incisions in humans and mice could be detected. Despite high compositional similarities (1159 PGs), only 50 proteins were commonly regulated across species (q-value <0.05, log2FC< [0.38]), but their direction of regulation was highly consistent. Moreover, Top10 reactome pathways of all regulated proteins were broadly comparable. Network analysis of human volunteers stratified as high responders revealed a pronounced proteolytic environment and elevated ROS-related proteins suggesting a prolonged inflammatory state. In contrast, low responder proteome signatures can be annotated to cell migration and anti-inflammatory processes.

Conclusion: Elucidation of altered proteins upon incision in human and mice. Furthermore, phenotyping of volunteers' identified responder type-specific protein alterations might present the underlying molecular fingerprint of hyperalgesic area expression. Network-based data analysis unraveled mechanistically relevant proteins in skin wound healing.

P08.13

Proteomic Signature Associated with Prognosis in HPV-Related Oropharyngeal Squamous Cell Carcinoma

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Introduction: Prediction of outcome in human papilloma virus (HPV)-related oropharyngeal squamous cell carcinomas (OPSCC) remains crude. New prognostic biomarkers are needed to rationalise curative-intent treatment and improve its efficacy. We aimed to develop a proteomic signature associated with risk of recurrence from diagnostic biopsies of patients with HPV-positive OPSCC.

Methods: We analysed 139 formalin-fixed paraffin-embedded (FFPE) archival core biopsy specimens from 124 patients with locally advanced HPV-positive OPSCC treated with chemoradiotherapy at the Princess Alexandra Hospital (Brisbane, Australia) 2007-2019. The cohort included 50 patients with recurrence less than five years from diagnosis (non-responders) and 74 age/performance-status matched responders. Proteomic analysis was performed utilizing data-independent acquisition mass spectrometry (DIA-MS).

Results: We quantified 4342 proteins with at least two peptides used to identify each protein. The median age was 60, 91% were male, and median follow-up was 64 months. 116 proteins were associated with recurrence free survival (RFS) on univariate analyses after adjusting for multiple comparisons. Upregulated proteins in the 116-protein signature among responders included signals of innate and adaptive immune activation. A 15-protein signature associated with 5-year RFS was derived by first ranking the proteins according to their significance in a univariate Cox model, and then using stepwise feature selection on top 50 proteins in a multivariate Cox model. The proteomic signature stratified patients into low, intermediate and high risk of recurrence and overall survival ($p < 0.0001$).

Conclusions: DIA MS-based proteomics on core biopsies can be used to risk stratify HPV-related OPSCC patients and identify patients at high risk of recurrence. Refining pre-treatment prognostication may inform future clinical trials to better tailor upfront therapy.

P08.14

Identification of Protein Biomarkers in FFPE Primary Tissues to Predict Recurrence in Endometrial Cancer

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Introduction: Endometrial cancer (EC) is the fourth most common cancer in women in developed countries and the sixth cause of death due to cancer. The clinicopathological classification, considered to be the gold standard, is inaccurate to predict tumor recurrence, which is the most important cause of death in EC patients. We aim to identify and verify predictive biomarkers of recurrence for different subtypes of EC, specifically, for endometrioid EC (EEC) at intermediate-to-high risk of recurrence, and for serous EC (SEC), which are at high risk of recurrence.
Methods: This study was approved by the Ethical Committee of each institution. FFPE primary tissues from a cohort of 102 patients including 64 intermediate-to-high risk EEC and 38 SEC were selected from Vall d'Hebron Hospital (Barcelona) and Arnau de Vilanova Hospital (Lleida). A discovery study comparing recurrent vs non-recurrent patients was performed using an untargeted label-free proteomic approach in the LTQ-Orbitrap Fusion Lumos. Verification of significant proteins was performed in FFPE primary tissues from an independent cohort of 129 EEC patients using a targeted approach (LC-MS PRM). Statistical analysis was performed using R script and p-values lower than 0.05 were considered statistically significant.
Results: A total of 4,569 and 5,747 proteins were detected in EEC and SEC patients. We identified 439 and 56 proteins differentially expressed in recurrent vs non-recurrent EEC and SEC patients, respectively. From those, 169 peptides from 58 proteins were studied in primary tissues of 129 EEC patients. Five proteins were verified with a p-value lower than 0.05.
Conclusions: We unveiled the proteomic landscape of recurrent EC and identified 5 protein biomarkers that could be potentially used as predictive biomarkers of recurrence for intermediate-to-high risk EEC. These results are aimed to improve the standard of care of EC.

P08.15

Protein Biomarkers in Pipelle Biopsies to Diagnose the Histological Type and Grade of Endometrial Cancer and Predict Tumor Recurrence

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Introduction: Endometrial cancer (EC) diagnosis relies on the observation of tumor cells in endometrial biopsies obtained by aspiration (i.e., pipelle biopsies, PB) but this procedure leads to 50% of incorrectly assigned EC histotype and grade. These prognostic factors are crutial to guide surgical treatment. Additionally, recurrence prediction is not accurately determined preoperatively. We aimed to identify protein biomarkers in the fluid of PB samples to overcome these limitations.

Methods: A spectral library was built by measuring by DDA a pool of 40 PB samples fractionated in 60 fractions. Then, a data-independent acquisition (DIA) approach was used for the identification and verification of biomarkers in PBs from 43 EC patients (discovery study) and 153 EC patients (validation study). Comparison groups were defined based on the tumor histological grade (low vs high grade patients), histological subtype (endometrioid EC vs non-endometrioid EC), and recurrence (recurrent vs non-recurrent EC). Data was analyzed using Spectronaut and R software.

Results: A spectral library of 5,863 proteins (54,448 peptides) detected in PB samples was generated. Among those, 96% overlapped with proteins identified in EC tissues from the Clinical Proteomics Tumor Analysis Consortium, confirming the usefulness of PB as a minimally-invasive source of EC biomarkers. After the discovery and validation studies, we identified 26, 28, and 19 differentially expressed proteins (adj.p-value<0.05, fold-change>2) between histologies, grades, and recurrent EC patients, respectively. Importantly, two proteins showed an AUC>0.85 for the discrimination of endometrioid EC and more aggressive non-endometrioid EC tumors in both cohorts. Another protein showed an AUC of 0.79 for recurrence prediction.

Conclusions: Here we present a unique spectral library of 5,863 proteins detected in PB samples that can be useful for other studies of gynecological diseases. Moreover, we identified promising biomarkers that will improve preoperative risk assessment in EC diagnosis to guide patients to an optimal surgical treatment.

P08.16

Immunopeptidomics-Based Development of a Listeria mRNA Vaccine

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Introduction: Antibacterial vaccines are recognized as effective tools to mitigate antibiotics resistance. For novel vaccines, knowledge about the identity of antigenic bacterial peptides presented on infected cells is essential, but missing for many bacteria. Listeria monocytogenes (Lm), as one of the major foodborne pathogenic bacteria, presents a particular threat to vulnerable individuals such as pregnant women, elderly people or immunocompromised patients and no commercial vaccine is available to date.
Methods: HeLa and HCT-116 cells, were infected with Lm and MHC class I-presented immunopeptides purified by immunoprecipitation followed by label-free and TMT-labeling LC-MS/MS analysis. Resulting data were searched using PEAKS studio and antigens giving rise to the highest number of presented bacterial immunopeptides were formulated as lipid vesicle-delivered mRNA vaccines. Levels of protection against Lm infection was assessed via prime-boost vaccination in C57BL/6J mice.
Results: Combining both infected cellular models and label-free with TMT-labeling data, 86 potential Listeria and 16,300 host immunopeptides were quantified. Predominance of 9mer peptides and matching peptide sequence-derived HLA motifs demonstrate characteristic immunopeptide features. Further filtering of the potential Listeria peptides yielded 68 high confidence Listeria immunopeptides from 42 antigens. Among the protein antigens of origin, eight were described already including prominent virulence factors such as LLO, plcA, plcB, inlB and actA. Interestingly, the antigen with most presented peptides was a membrane-bound antigen not described as immunodominant previously. Vaccination with the top antigens resulted in high levels of protection for several antigens including the novel membrane-bound antigen.
Conclusions: Combining label-free and TMT-labeling approaches in immunopeptidomics on multiple cellular models facilitated the identification of novel, immunogenic and protective Listeria antigens. This knowledge might be further used to develop a Listeria vaccine or immunological therapeutics. Furthermore, the applied pipeline may also serve to explore immunogenic antigens of other pathogenic, intracellular bacteria.

P08.17

Regulation of Protein Cargo in Extracellular Vesicles during Cancer Onset

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Introduction: Exosomes are micro vesicles produced by most eukaryotic cells as a normal physiologic process. In the past few years there has been an increasing interest in these vesicles since they have been related to functions like coagulation, inter-cellular signaling, T-cell activation and transferring antigens to the surface of professional antigen presenting cells. Particularly, there are reports of changes in the composition of exosome cargo during the development of different types of cancer.
It has been described that exosomes generated by malignant cell lines and isolated from cancer patients, carry a higher content of proteins related to extracellular matrix remodeling, angiogenesis, organotropism, chemoatraction and mediators of epithelial-mesenchymal transition. However, the mechanisms used by tumors to modulate exosomal cargo of proteins in a way that benefits cancer progression is yet to be described.
This study aims to describe how the balance of different post-translational modifications competing for the same target amino-acid, regulate the translocation of proteins into exosomes produced by cellular models including cervix, breast and lung cancer. Here we show some preliminary results of this project in progress.
Methods: Cells were challenged by the inhibition of different pathways to reduce or increase the abundance lysine acetylation and ubiquitination. Exosomes were enriched by a combination of ultracentrifugation and filtration. Characterization was carried out using flow cytometry and LC-MSMS analysis of cargo proteins.
Results: Both, the intracellular proteins and the cargo of extracellular vesicles, modified their expression levels in response to the variation of the abundance in the mentioned PTMs. Vesicular cargo alterations included several proteins involved in redox homeostasis, carbon metabolism and organization of the cytoskeleton.
Conclusions: the alteration of the protein expression profiles in the content of extracellular vesicles can indicate the status of the PTMs inside the cells and could be used to predict the outcome of targeted treatments.

P08.19

Evaluation of Melanoma Plasma Proteome Profile and the Modulation of Plasma Proteins Based on Tumor Proliferation

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Introduction: The analysis of plasma from melanoma patients supports the diagnosis and prognosis of malignant melanoma (MM). Efforts are currently being made for discovering new and more accurate plasma biomarkers. In this work, we aimed to characterize for the first time the plasma proteome profile of MM patients in different stages of the disease using sample depletion approaches and high-resolution mass spectrometry. Methods: Here we analyzed total plasma and applied depletion strategies using top7, top14 (Multiple Affinity Removal Column Human 7 and 14, Agilent), and Supermix columns (Seppro® SuperMix LC2, Sigma). The samples were submitted to a simple digestion protocol using Lys-C and Trypsin in S-Trap™ sample processing technology (PROTIFI). Results: More than 1,000 proteins were identified. In the analysis of pooled plasma of MM patients, we were able to cover different proteins classes in a wide range of plasma protein abundance, including 63% of the approved FDA biomarkers. Most of the proteins identified have previously been identified in exosomes. The role of these membrane-bound extracellular vesicles has been extensively studied in cancer and there is great potential as a biomarker. In the analysis of MM patients, the samples were discriminated into two groups based on the proliferation status according to the sPLS-DA analysis. We selected the top 100 proteins that drive this discrimination, and we could see the deregulation of the immune system, extracellular organization, acute-phase, cell migration, and apoptosis, for instance. Potential serum/plasma biomarkers such as LDH, Serum amyloid, and CRP were identified upregulated in the medium-high/high proliferative groups, which indicates a correlation to tumor cell proliferation. Conclusions: In this work, we had great coverage of plasma proteome being able to identify important biomarkers for cancer and other pathologies. Moreover, we could see an influence of tumor proliferation on the modulation of the immune system.

P08.20

Diagnosis of Pleural Effusions Using Mass Spectrometry-Based Targeted Proteomics

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Introduction: Pleural effusion (PE) is a pathological accumulation of pleural fluid in the pleural cavity. Its removal is required and is minimally invasive. Many diseases may cause PEs, the most common being heart failure, malignancy, pneumonia and tuberculosis (TB). Treatment and prognosis of PE depend on its cause. Determining the etiology of a PE in a quick and non-invasive manner is currently a challenge due to poor sensitivity of diagnostic methods. 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 discriminates benign, malignant, tuberculous and other-infectious-PEs.

Methods: Our developed panel consists of 34 proteins, which are cancer, TB and other pleural/pulmonary infectious disease markers currently used in the clinic, as well as suggested biomarkers from the research literature. The sample preparation method was optimized and a Multiple Reaction Monitoring (MRM) assay was developed with 105 stable-heavy-isotope-labeled internal standard peptides. 209 PE samples collected from patients during thoracentesis were analyzed using the developed targeted proteomic method by nano-liquid chromatography (LC)-triple quadrupole (TQ)-mass spectrometry (MS).

Results: We detected 31 out of 34 proteins being relevant for PE classification. Four proteins specifically discriminate malignant-PEs (MPEs) from the other PEs, three proteins specifically discriminate tuberculous-PEs (TPEs) from the other PEs, and seven proteins specifically discriminate the all-infectious-PEs group (TB and other-infectious-PEs) from the other PEs.
A four-group classifier tool shows a very good discrimination ability for classifying PEs into one of the four PE types: AUC of 0.863 for MPEs, AUC of 0.859 for TPEs, AUC of 0.863 for other-infectious-PEs, and an AUC of 0.842 for benign-PEs.

Conclusions: The developed panel could potentially be useful for discriminating benign, cancerous, tuberculous and other-infectious-PEs, as a less-invasive, supportive diagnostic tool to shorten PE diagnosis time.

P08.21

An 8-Channel Automatic Glycan Profiling System Realized by The GlycoBIST Technology

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Introduction: Recent outcomes on the omics-based discovery of disease-relevant glyco-biomarker have steadily led to translational research for clinical diagnosis. Lectin microarray has been recognized as an outstanding supporting technology that can find glycan differences using trace amounts of clinical specimens. To accelerate the validation, we introduced a “lectin bead array in a single tip” (GlycoBIST) as a potential automatic glyco-analysis technology (1), whose concept was reported at the HUPO 2015. In this congress, we report on the successful fabrication of an 8-channel automated system.

Methods: Every step on beads filled in a GlycoBIST tip was carried out by a prototype auto-machine LuBEA-GT VIII, which is composed of an eight-channel auto-pipetting machine, an 8-lined reaction-cartridge holder, and a multiplex chemiluminescent scanner (2). The validity was evaluated by comparing the glycan profiles with a manual system (1).

Results: We preliminarily verified the simultaneous signal repeatability of lectin-bead with a CV of <10% using 90 of 1,000 bead stock simultaneously coated with each lectin. Using a GlycoBIST tip including up to 15 lectin-beads selected from >30 species of the lectin-bead lineups, we automatically acquired the glycan profile for nanogram-quantities of each glycoprotein within 50-min by LuBEA-GT VIII. The resultant data was consistent well with that of the manual system.

Conclusions: Quick differential glycan profiling among eight samples could be realized by LuBEA-GT VIII with GlycoBIST tips comprising 15-plexed lectin beads. The “minimized” glycan profile is beneficial not only for the glycobiomarker validation but as a highly versatile glyco-test just for non-glyco-researchers.
This study was supported by projects for utilizing glycans in the development of innovative drug discovery technologies from the Japan Agency for Medical Research and Development (AMED).

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P08.22

Proteogenomics for Splicing Variation and Differential Expression: A Myotonic Dystrophy Type 1 Mouse Model Study

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Introduction
Dysregulated mRNA splicing is involved in the pathogenesis of many diseases including myotonic dystrophy type 1 (DM1). Comprehensive assessment of dysregulated splicing on the transcriptome and proteome level has been methodologically challenging, and thus investigations often target only a few select genes. We performed a large-scale coordinated transcriptomic and proteomic analysis to characterize a DM1 mouse model (HSALR). Our integrative proteogenomics approach comprised gene- and splicing-level assessments for mRNAs and proteins.
Methods
Paired-end transcriptome sequencing was performed with HiSeq 4000 (Illumina) generating 71 million reads per sample on average. Reads were aligned using STAR v2.5.2a (Dobin et al., 2013), statistical analysis of gene expression and differential alternative splicing were performed using R/Bioconductor and LeafCutter v0.2.7 (Li et al., 2018), respectively. For proteomic analysis, samples were fractionated into 24 fractions and analyzed with Orbitrap Fusion Lumos using TMT-based approach. In total, more than 53 000 peptides and 5800 protein groups were identified and quantified. For splicing-specific analysis, we performed targeted analysis (PRM) for 100 peptides using Orbitrap QExactive HF-X. Data analysis was performed using Thermo Proteome Discoverer, Skyline, and custom Python scripts.
Results
The transcriptomic analysis recapitulated many known instances of aberrant splicing in DM1 and identified new ones. It enabled the design and targeting of splicing-specific peptides and confirmed the translation of known instances of aberrantly spliced disease-related genes (e.g. Atp2a1, Bin1, Ryr1), complemented by novel findings (e.g. Ywhae, Flnc, Svil). Comparative analysis of large-scale mRNA and protein expression data showed remarkable agreement on both the gene (Pearson correlation 0.91 for significantly changed genes) and especially the splicing level (Pearson correlation 0.95).
Conclusions
We believe that our work is suitable as a model for a robust and scalable integrative proteogenomic strategy. This strategy advances our understanding of splicing-based disorders, and helps establish robust splicing-specific biomarkers.

P08.24

Discovery of Soluble Pancreatic Cancer Biomarkers Using Innovative Clinical Proteomics and Statistical Learning.

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) late diagnosis is primarily attributed to its asymptomatic progression combined with absence of any reliable screening markers. This makes PDAC one of the deadliest cancer with a 5-years survival rate less than 10%. Gold standard diagnosis is provided by endoscopy-guided fine-needle biopsy (EGFNB), although it is invasive, risky and with a poor level of negative predictive value (NPV).
Methods: In this proof-of-concept study, we developed a novel proteomic approach which recovers the soluble proteins in the EGFNB that remains a rich source of potential biomarkers (1) without conflicting with the usual diagnostic procedure. Proteomic analysis of the soluble proteins led to over 2500 identifications, which were subjected to subsequent statistical analysis. To build the subsequent protein signature score (PSS), we used several resampling methods (2) at different steps of the analysis and an algorithm derived from microarray analysis techniques (3).
Results: We followed 58 patients that underwent pancreatic EGFNB, of which 43 were diagnosed as PDAC while 15 had non-cancerous lesions. The PSS achieved 0.917 and 0.853 of sensitivity and specificity rates respectively. We then linked the PSS with clinical data to provide a decision algorithm achieving 100% of positive predictive value and 92.3% of NPV.
Conclusions: The remaining EGFNB fluid is a rich reservoir of proteins capable of identifying PDAC among patients with cancerous or non-cancerous pancreatic masses. Due to their soluble nature, the newly discovered protein biomarkers bare the potential to be detected in the patient serum. This will enable the development of non-invasive blood-sample based assays to a larger patient cohort, leading to the hope of promoting a population-based screening test, allowing for quicker management at an earlier stage.

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P08.25

Discovery of Candidate Stool Biomarker Proteins for Biliary Atresia Using Deep Proteome Analysis by Data-Independent Acquisition Mass Spectrometry

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Background: Biliary atresia (BA) is a destructive inflammatory obliterative cholangiopathy of the neonate that affects various parts of the bile duct. If early diagnosis followed by Kasai portoenterostomy is not performed, progressive liver cirrhosis frequently leads to liver transplantation in the early stage of life. Therefore, prompt diagnosis is necessary for the rescue of BA patients. In this study, we discovered potential biomarkers for BA using deep proteome analysis by data-independent acquisition mass spectrometry (DIA-MS).
Method: Four BA patients before Kasai portoenterostomy and three non-BA patients were recruited for stool proteome analysis. Soluble proteins were collected from stools and enzymatically digested. The digested peptides were analyzed by DIA LC-MS/MS (Q-Exactive HF-X with UltiMate 3000 RSLCnano system, Thermo Fisher Scientific). Proteins were identified and quantified by Scaffold DIA software (Proteome Software Inc.) from MS data. To determine differential proteins between BA and non-BA, the statistical p-value (Mann-Whitney U test, $p < 0.05$) was used in data analysis.
Result: 2,110 host-derived proteins were identified. The host stool proteins overlapped only approximately 50% with the plasma proteins, and the plasma and stools had different protein profiles. Among the identified proteins in stools, 103 proteins were significantly different ($p < 0.05$) between the two groups (BA vs. non-BA). Of these 103 proteins, 49 proteins were significantly higher in patients with BA, whereas 54 proteins were significantly lower in patients with BA.
Conclusion: Our study is the first to establish deep proteome analysis of stools and apply it to infants with cholestasis, including both BA and non-BA cohorts. Our new method of deep proteome analysis by DIA-MS can detect over 2,000 host-derived proteins in stools and provides a method for discovering new BA biomarkers. Deep proteome analysis of stools has great potential to elucidate the pathophysiology of BA and other diseases, especially in the field of gastroenterology.

P08.26

Large scale, deep and unbiased plasma proteomics profiling a sub-study of a multi-cancer cohort enabling biomarker discovery

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Over the past 20 years, proteomics has vastly increased our knowledge of biology, yet the translation to commercial clinical tests assisting physicians with better treatment strategy for patients is very limited. The primary technical challenges in translating Liquid Chromatography coupled to tandem Mass spectrometry-based proteomics (LC-MS/MS) discoveries to the clinic have historically been reproducibility, throughput combined with proteome depth and accessibility of large clinical cohorts. Recent advances in sample preparation (i.e. Seer's ProteographTM Product Suite) coupled with improved mass spectrometry instrument sensitivity and speed, are providing the ability to quantify thousands of proteins from human plasma without compromising throughput or reproducibility, thus creating a unique opportunity to detect robust protein biomarkers which translate to viable clinical test for complex diseases.

Human K2EDTA plasma samples were prospectively collected following an IRB approved protocol and processed for LC-MS/MS utilizing Seer's Proteograph Assay. LC-MS/MS data were analyzed utilizing MaxQuant with Match-Between-Runs (MBR) and Spectronaut, and statistical analysis performed with R and Python.

A prior plasma study1 on average identified 1,664 protein groups per subject. Using the five-nanoparticle panel workflow, we are reporting on a high-throughput cancer proteomics biomarker study on a pilot study of 212 subjects, we identified ~2,000 protein groups per subject with low median CVs and ~5100 protein groups across 240 samples (including controls). Herein, we report on this cohort and demonstrate deep unbiased proteomics with high reproducibility, and data collection throughput.

We reported on a plasma proteomics biomarker discovery study at unprecedented scale and depth, with reproducibility and statistical power to address historical technical challenges in translating proteomics to the clinic. Our approach also captures post-translational modification (PTM) known to play a role in cancer pathogenesis providing data that could reveal fundamental new molecular insights into cancer.

P08.27

Unbiased High-Throughput Mass Spectrometry-Based Plasma Proteomics for Detection of Early Stage Lung Cancer

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Introduction: Early lung cancer detection saves lives, and there is high demand for cost-effective blood-based screening methods to enable early diagnosis of the disease and to support other early detection methods such as low dose CT (LDCT) scanning. We have used high-throughput mass spectrometry-based proteomics to analyze plasma samples from early stage (stage I and II) adenocarcinoma lung patients and control samples from age and smoking history-matched cancer-free donors to validate the potential of plasma proteomics to identify biomarkers for early lung cancer detection against a clinically relevant background cohort.

Methods: 42 plasma samples were analyzed including 17 from cancer patients (82 % stage I and 18 % stage II). Samples were collected at the Massachusetts General Hospital. Digested samples were separated by nano HPLC (nanoElute, Bruker) using 17.8 gradients connected to a timsTOF Pro, (Bruker Daltonics) operating in dia-PASEF mode. Data were processed with Spectronaut software. A random forest progressor was used to identify multi-biomarker sets using 100 different dataset groupings with a 20 % holdout and 80 % used for training.

Results: Label-free DIA analyses of the plasma samples using a 30 min method (a throughput of 50 samples per day) resulted in an average quantification of 390 protein groups per sample covering over 5 orders of dynamic range. Random forest-based classification of plasma proteome data resulted in a receiver operating characteristic (ROC) curve area of 0.933 showing excellent classification power. Using this biomarker discovery set, we can correctly retain 7 of every 10 early-stage cancer patients for work-up/surgery following a positive LDCT, while only falsely diagnosing cancer in 1 out of 20 controls (70% sensitivity at 95 % specificity).

Conclusions: DIA plasma proteomics is showing great potential for accurate and cost-effective detection of early stage lung cancer.

P08.28

Proteomic Characterization of Primary Tumors and Brain Metastases in Lung Adenocarcinoma Patients

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Introduction
Lung adenocarcinoma (LADC) is the most common subtype of non-small cell lung cancer (NSCLC). One major complication during disease progression is the metastatic spread to the brain. Treatment regimens for brain metastases are limited, thus these metastases remain the leading cause of tumor-associated deaths globally. The aim of this study was to investigate the proteomic differences of primary LADC and its brain metastases with reference to fast versus slow metastasis development.

Methods
A total of 20 LADC patients with brain metastases were included in this study. Histopathological parameters and patient characteristics were assessed and included in further analysis. Proteomic profiling was conducted on FFPE tissue samples via nLC-MS/MS analysis (Ultimate 3000 RSLC nano pump coupled to Q-Exactive HF-X MS) using label free quantification, followed by database search and statistical evaluation (Proteome Discoverer 2.4, Perseus, RStudio, Graphpad Prism).

Results
Protein- and pathway-based comparisons revealed several differentially expressed proteins and pathways which may promote metastasis to the brain. Protein profiles characteristic of primary and metastatic tumors as well as proteins associated with tumorigenesis were identified. Of note, proteins and pathways negatively regulated in patients with faster progression to brain metastasis were frequently associated with cell-cell interactions and extracellular matrix. These data might indicate a malfunction of cellular attachment reinforcing metastasis to the brain. Additionally, the ribosome pathway was prominently upregulated in primary tumors of fast progressing patients as well as in patients who developed multiple brain metastases.

Conclusions
This study is the first comprehensive proteomic analysis of paired primary tumors and brain metastases of LADC patients. Insights on proteomic differences between fast and slow progressing patients may provide biomarkers for stratification of LADC patients and contribute to the understanding of intracranial metastasis development.

P08.29

A Pan-Cancer Proteomic Map of 960 Human Cell Lines

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Introduction: Proteomic data provide unique insights into the molecular behaviour of cells in both healthy and disease contexts. Proteomics can reveal novel associations between genotype and phenotype, beyond what is apparent from genomics or transcriptomics alone. However, a lack of large proteomic datasets across a range of cancer types has limited our understanding of proteome network organisation and regulation.

Methods: We produced a pan-cancer proteomic map derived from 960 human cancer cell lines. The map encompasses more than 40 cancer types derived from over 28 distinct human tissues. The samples were processed with a clinically-relevant workflow involving rapid and minimally complex sample preparation. The raw proteomic data were acquired by data independent acquisition mass spectrometry (DIA-MS). The processed data were analysed with a bespoke deep learning-based pipeline (DeeProM) that integrates multi-omics, CRISPR-Cas9 gene essentiality and drug sensitivity information.

Results: There are three major outcomes. First, our findings reveal pervasive post-transcriptional modification and thousands of putative protein biomarkers of cancer vulnerabilities. Second, DeeProM statistics show that a fraction of the proteome can confer similar predictive power to the entire transcriptome. This has key implications for the clinical application of proteomics in drug response prediction. Third, we demonstrate that a random proportion of the identified proteins can provide robust predictions of cancer cell phenotypes, underpinning the concept of pervasive co-regulation of protein networks.

Conclusions: This pan-cancer cell line proteomic map is a comprehensive resource that expands our understanding of cancer proteomes. These DIA-MS data reveal principles of cancer cell phenotypes, including genetic vulnerabilities and drug sensitivities, that are important for developing novel targeted anticancer therapies.

P08.31

Accurate Quantitation of Clinically Approved Cancer Biomarkers Utilising SRM

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Introduction: Compared to immunohistochemistry (IHC), selected reaction monitoring (SRM) has several advantages for routine clinical testing. SRM is capable of highly reproducible identification and quantitation of protein biomarkers over a larger dynamic range, with high target specificity and sensitivity, and is suitable for rapid processing of multiplexed panels. Our aim is to evaluate the use of SRM assays in clinical oncology.
Methods: We have developed SRM assays for 63 cancer protein biomarkers which are currently detected by IHC in pathology laboratories. Seven of these (HER2, estrogen receptor, progesterone receptor, BRCA1, BRCA2, EGFR and Ki67) are currently in use for breast cancer diagnosis and treatment selection, and 52 are used in the diagnosis of cancer of unknown primary (CUP). A minimum 3 peptides per protein with an average of 8 transitions per peptide were used for quantitation. Assays were performed on a Sciex QTRAP 5500 with 20 min acquisitions using microflow LC, quantifying ~25 proteins per run.
Results: The breast cancer SRM screen was tested against a breast cancer cell line panel with known expression levels of the 7 biomarkers and response to chemotherapeutics. Six of the biomarkers were accurately quantitated with SRM, corresponding with expected protein expression levels and IC50 values for HER2 inhibitor and hormone therapies. The 52 CUP SRM assays were evaluated on a panel of patient samples representing 22 cancer types.
Conclusion: Our study demonstrates that biomarkers already utilised in the clinic can be accurately quantitated with SRM and highlights the feasibility of significantly expanding the use of SRM within a clinical setting. The accurate quantitation SRM provides may ultimately be utilised by clinicians to better stratify patients for likely therapeutic response and lead to improved selection of treatment strategies.

P09.01

Proteomics of the Acquired Resistance to Targeted Kinase Inhibition in Pancreatic Cancer Cells

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Introduction: Pancreatic cancer is one of the most worrying neoplasms with a 5-year survival rate of less than 7%. Existing treatments are not effective to alleviate poor prognosis, consequently, new alternative therapies are highly welcome. Targeted molecular therapy directed to pivotal signaling pathways sustaining the proliferation of pancreatic cancer cells has emerged as an attractive therapeutic approach. Blockade of mutant KRas signaling by inhibition of downstream Raf/MEK/ERK and PI3K/Akt pathways has been tested in preclinical models, but results have been disappointing. Indeed, although such pathways are crucially related to cell cycle progression, cell growth and survival, acquisition of resistance to therapeutic drugs represents a difficult and frustrating phenomenon.

Methods: Previously identified as MEK and PI3K kinase inhibition-sensitive cells, KRas-mutant MIA PaCa-2 pancreatic cancer cell line was cultured in medium with corresponding kinase inhibitors for several months until proliferation rates were equivalent to those of parental cells. We carried out a data-independent acquisition-mass spectrometry analysis of the cell proteomes, using two peptide fractionation methods (strong anion-exchange and high pH reverse phase microcolumn chromatography). Finally, protein differential expression between resistant and parental phenotypes was determined.

Results: More than 4 300 proteins were relatively quantitated. Proteome comparison shows that MEK and PI3K kinase inhibition-resistant MIA PaCa-2 cells overexpress the KRas oncoprotein and some members of S100 protein family which are known to be related with progression and poor prognosis in pancreatic cancer, including S100A2 and S100A16. Distinct metabolic profile between both cell phenotypes was also observed. Moreover, resistant cells maintain phosphorylation of MEK and PI3K kinase substrates ERK and Akt proteins.

Conclusions: The proteome of pancreatic cancer cells with acquired resistance to targeted inhibition of MEK and PI3K kinases is linked to new relevant candidate drug targets which are of great interest for further validation and research.

P09.02

Proteomic Characterization of two Extracellular Vesicle Subtypes Isolated from Human Glioblastoma Stem Cell Secretome by Sequential Centrifugal Ultrafiltration

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Introduction:Extracellular vesicles (EVs) are today actively investigated since molecules identified in their content may represent new cancer biomarkers and/or druggable targets in case they are transferred to neighboring cells, supplying them with oncogenic information/functions. EVs, including endosome -derived exosome (Exos, 30-100nm size)and plasma membrane-derived microvescicles (MVs,100-1000 nm size), secreted by all cell types well refelect the identity and the molecular state of their cell of origin (1,2). Especially in cancer EVs can mirror its peculiar characteristics. This aspect seems of particular relevance, since the identification of molecules inside EVs, which could be transferred to neighbor cells helping them in growing and potentially increasing their malignancy, could allow to identify new tumor markers.
Methods: we characterize by a proteomic point of view two subtypes of EVs isolated by sequential centrifugal ultrafiltration technique from culture medium of stem-like cells (GSCs) obtained from surgical specimens of glioblastoma (GBM), the most aggressive and lethal primary brain tumor in humans. Electron microscopy and western blot analysis distinguished them into microvesicles (MVs) and exosomes (Exos). Two-dimensional electrophoresis followed by MALDI TOF analysis allowed us to identify, besides a common pool, sets of proteins specific for each EV subtypes with peculiar differences in their molecular/biological functions.
Results: such a diversity was confirmed by the identification of some top proteins selected in MVs and Exos. They were mainly chaperones or 34 metabolic enzymes in MVs whereas, in Exos, molecules are involved in cell-matrix adhesion, cell migration/aggressiveness and chemotherapy resistance.
Conclusion:if our data will be confirmed in EVs isolated from a greater number of GSCs deriving from different primary human tumors, proteins here analyzed could be regarded as new possible GBM prognostic markers/druggable targets, thus opening new perspectives for therapy.

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P09.04

DIA-MS Identifies and Validates Transgelin as Protein Contributing to a Poor Response of Metastatic Renal Cell Carcinoma to Sunitinib Treatment

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Introduction. Renal cell carcinoma (RCC) represents about 2–3% of all cancers with over 400,000 new cases per year. Sunitinib, a vascular endothelial growth factor tyrosine kinase receptor inhibitor, has been used mainly for first-line treatment of metastatic clear-cell RCC (mccRCC) with good or intermediate prognosis. However, about one-third of mccRCC patients do not respond to sunitinib, leading to disease progression.
Methods. Here, we aim to find and characterize proteins associated with poor sunitinib response. Sixteen RCC tumors from patients responding (8) vs. non-responding (8) to sunitinib 3 months after treatment initiation were analyzed using data-independent acquisition mass spectrometry (DIA-MS) on Impact II LC-MS system (Bruker), together with their adjacent non-cancerous tissues in a pilot proteomics study. Validation was performed in an independent cohort of 75 mccRCC patients using DIA-MS on QExactive HF-X (Thermo Fisher Scientific), with data analysis in Spectronaut 13.9 (Biognosys). Transgelin protein role was functionally analyzed in RCC cell lines using CRISPR and RNAi silencing techniques.
Results. Proteomics analysis quantified 1996 protein groups (FDR=0.01) and revealed 27 proteins deregulated between tumors non-responding vs. responding to sunitinib in the pilot study, representing a pattern of proteins potentially contributing to sunitinib resistance. Validation cohort confirmed up-regulation of transgelin in tumors non-responding to sunitinib and revealed its association with tumor grade. Gene set enrichment analysis showed an up-regulation of epithelial-to-mesenchymal transition with transgelin as one of the most significantly abundant proteins. Reduced transgelin protein level in CRISPR and RNAi altered RCC cells led to significantly slower proliferation of these cells and affected their survival.
Conclusion. Altogether, our data indicate that transgelin is an essential protein supporting RCC cell proliferation, which could contribute to intrinsic sunitinib resistance, and is associated with aggressive phenotype of RCC tumors.
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P09.06

Efficient Profiling of Protein Degraders by Specific Functional and Target Engagement Readouts

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Introduction
Targeted protein degraders such as molecular glues and proteolytic targeting chimeras (PROTACs) opened a whole world of modalities, when specially designed small molecules bring together enzyme and the protein of interest (POI). In the same way therapeutic targets could be subjected to other enzymatic reactions, by different TACs. In order to characterize the mechanism of action of such compounds, one need to study binding specificity towards the recruited enzyme, target proteins, as well as confirm therapeutic action in the relevant sample matrix (e.g. protein degradation).
Methods
The Cellular Thermal Shift Assay (CETSA) in combination with mass spectrometry (MS) allows to assess thousands of proteins in a complex sample matrix and identify those that change either thermal stability or abundance (or both) upon compound treatment. In this way the method can serve as a perfect tool for extensive characterization of targeted protein degraders.

Results
We have used CETSA MS to profile several commercially available protein degraders, covering the most frequently used E3 ubiquitin ligases, multiple target proteins and different linkers used to build the PROTAC molecule. Our aim has been to understand mode of action and to deconvolute off-target effects in cells by the specific compounds or specific parts of the PROTAC molecules, e.g. E3 ubiquitin ligase ligand, the warhead and the linker. As an example of non-chimeric protein degrader, we have studied pomalidomide-induced changes in protein abundance and stability changes at different time points, focusing on the binding and degradation specificity.
Conclusions
In addition to the stabilization of the recruited enzyme and degradation of the targeted proteins, a number of off-target events were observed, including off-target degradation and off-target inhibition of various proteins.

P09.07

Label Free Pharmacoproteomic Assays Enabled the Discovery of Cellular Pathways Involved in the Survival Of MCF-7 and K562 Cancer Cells

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Introduction
A higher toxicity of DMC for human cancer cells with or without a functioning p53 has been investigated by independent research groups. To better understand the mechanisms underlying DMC and MC toxicity we employed global and label free quantitative (LFQ) proteomics assays for profiling the changes in the proteomes associated with the cellular signaling networks modulated by the two drugs in MCF-7 (p53-proficient), and K562 (p53-deficient) in comparison with control, untreated cells.

Methods
Human breast cancer cells (MCF-7) and leukemia cancer cells (K562) were treated with 50 µM MC or DMC for 24 hours at 37oC. The proteomics platform employed nanoLC/MS/MS sequencing of tryptic peptides from in solution digests of total cell lysates using a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the label free quantification (LFQ) method for data analysis. The MS/MS spectra were searched with PEAKS and Scaffold proteome suite. The ingenuity pathway analysis (IPA) and REACTOME were used to map the GO annotations and assess the quantitative changes in the cellular proteomes.

Results
The global proteomics analysis retrieved about 2300-2600 proteins (FDR <1.2% for proteins) for each sample. The bioinformatics analysis predicted that MC and DMC can significantly increase cell death of MCF-7 and K562 cells. This was projected to be mostly accomplished by coordinated inhibition of cell survival and proliferation, mainly mediated by a downregulation of mTOR, Ras/Pi3K/Akt, EIF2/protein translation and JAK/STATs/NF-kB signaling pathways; and downregulation of proteins involved in the DNA repair machinery. Protein arrays and western blotting validation of pi3K/Akt, JAK/STATs/NF-kB and Ras/MAPK-related pathways validated some significant findings from the proteomics data.

Conclusions
The LFQ proteomics confirmed that integrative cellular and “omics” assays are valuable tools for providing a deeper understanding of the cytotoxicity of MC and DMC in p53-proficient and p53-deficient cancer cells in relation to the structure of both drugs.

P09.09

Proteomic Analysis of Equine Serum Antibody Repertoire against Loxosceles Venom.

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Introduction: Loxoscelism is defined as the sum of clinical manifestations caused by the brown spider (genus Loxosceles) bite that affects around 8,000 people in Brazil annually. The anti-venom serum produced in horses is the only specific treatment for the severe accidents despite its disadvantages. This therapy demands a large quantity of venom for immunization, it is harmful for the horses and it may cause allergic reactions. Therefore, several groups have attempted to overcome these limitations through the use of recombinant antigens or the production of monoclonal antibodies. Here, we aim to study the antigen-specific equine antibody repertoire to contribute to improve anti-venom therapy. Methods: Serum immunoglobulin from four horses hyperimmunized with Loxosceles venom were isolated, cleaved with pepsin and the F(ab')2 fractions were enriched against a pool of venoms from three Loxosceles species or against recombinant L. intermedia sphingomyelinase D (LiD1). The specific F(ab')2 fractions were digested with trypsin and submitted to LC-MS/MS. The data was analyzed with the software Proteome Discoverer (Thermo) using personalized BCR-Rep-Seq database for each horse. The identified peptides were mapped to antibody regions and those from the third complementarity determinant region of the heavy chain (CDRH3) were used to antibody identification. Results: We identified 12 specific antibody sequences against LiD1 and 20 against the pool of venoms, being 7 sequences common between both antigens. These sequences contained CDRH3 length ranging from 6 to 24 amino acids, with an average length of 17. The immunoglobulin genes IGHV4-29 and IGHJ6-1*01 were used in 93.75% of the antibodies identified. Regarding the identified immunoglobulins' isotype, 29 (90.6%) of them were IgG and 3 (9.4%) were IgM. Conclusions: These results improve the knowledge of the antigen-specific repertoire against spider venom and is a key step for future production of recombinant antibodies to compose a synthetic polyclonal treatment for loxoscelism.

P09.10

Discovering Substrates of PRMT5 and CDK4/6 In Human Melanoma Cells with Antibody-Based PTM Peptide Specific Enrichment Strategies.

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Introduction: The targeting of the cell cycle dependent kinases CDK4/6 with the small molecule inhibitor palbociclib has proven to be a successful anticancer treatment for a number of solid cell cancers. Moreover, inhibition of the protein methyl transferase PRMT5, which catalyzes the formation of symmetric dimethyl arginine (SDMA) with the compound GSK3326595 has also been identified as a promising anticancer treatment. This study identifies and quantifies the reduction in phosphorylation for the targets of MAPK/CDK kinases inhibited by palbociclib, and the SDMA sites down regulated with GSK3326595 inhibition of PRMT5 treatment.

Methods: The human melanoma cell line A375 was treated with the vehicle control (DMSO), the CDK4/6 inhibitor Palbociclib, the PRMT5 inhibitor GSK3326595, or both compounds together for 24 hours in triplicate experiments. The sites of MAPK/CDK phosphorylation and PRMT5 methylation were identified by sequential enrichment of tryptic peptides from the treated cells using antibodies specific for substrates of MAPK/CDK phosphorylation and SDMA containing peptides prior to LC-MS/MS. Changes in peptide abundance between treated and control was determined by label-free quantitation of peptide intensity in the MS1 channel using Skyline.

Results: Following SDMA specific peptide enrichment and LC-MS/MS we identified over 300 SDMA sites on over 250 proteins, with greater than 30 sites showing a four-fold or more reduction in abundance with GSK3326595 treatment. The use of MAPK/CDK substrate specific antibodies combined with label-free quantitation showed that Palbociclib treatment led to the significant reduction in phosphorylation of hundreds of sites of S/T threonine phosphorylation on proteins cell wide.

Conclusions: Antibody-based PTM peptide enrichment can specifically enrich for sites of arginine methylation, distinguishing symmetric from asymmetric arginine methylation allowing for the identification of PRMT5 substrates. Sites of phosphorylation inhibited by Palbociclib treatment were identified and quantified using the orthogonal strategies of substrate specific phospho-motif antibodies and IMAC.

P09.11

Improved Middle-down Characterization of Antibodies Using Proton Transfer Charge Reduction on a Tribrid Orbitrap Mass Spectrometer

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Introduction: Monoclonal antibodies (mAbs) represent the leading class of biotherapeutics. Mass spectrometry (MS)-based analysis is fundamental for both the development and quality control stages to ensure product quality. The middle-down (MD) approach, based on IdeS proteolysis, ensures the generation of mAb subunits in the 25-100 kDa mass range with minimal risk of introducing artificial modifications to the sample. Here we demonstrate the progress in MD MS analysis allowed by the application of proton transfer charge reduction (PTCR) on a Thermo Scientific Orbitrap Eclipse mass spectrometer.

Methods: mAbs of Ig1 and IgG4 subclasses and an ADC mimic were purchased from NIST and Millipore Sigma. Samples were digested using Ides (FabRICATOR, Genovis). Digestion products were analyzed with (to obtain ~25 kDa subunits) or without (~100 kDa F(ab')2) denaturation/disulfide bond reduction. Subunits were separated by reversed-phase liquid chromatography (RPLC). Fragmentation was performed using electron transfer dissociation (ETD), higher-energy collisional dissociation (HCD), their combination (EThcD) or 213 nm ultraviolet photodissociation (UVPD). Each dissociation technique was applied alone (MS2 experiments) or followed by PTCR (MS3 experiments).

Results: The application of EThcD in RPLC-MS2 experiments typically allowed for >50% sequence coverage on mAb subunits in a single run. However, the application of PTCR led to an increase in sequence coverage, number of identified fragment ions and of matched complementary ion pairs. For example, the Fd subunit typically moves from ~55% coverage of EThcD MS2 experiments to ~65% of EThcD MS2 – PTCR MS3 runs. Similarly, the F(ab')2 subunit of NIST mAb passed from ~17% sequence coverage to ~22% sequence coverage through the application of PTCR after ETD. Similar increases were observed for all the tested ion activation methods.

Conclusions: PTCR reproducibly improved the characterization of mAbs and ADCs, while complementary fragment ion pairs elevated the confidence in backbone cleavage assignment.

P09.12

Combatting Fungal Infections: Novel Anti-virulence Strategies and Reversing Antifungal Resistance.

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Introduction: Fungal pathogens are critically important threats to global health with over 300 million people affected by serious fungal diseases worldwide. In Canada, pathogenic fungi are a growing public health concern with the evolution of drug-resistant strains and the emergence of new pathogens. Fungal pathogens are a leading cause of human mortality, particularly among the ever-increasing population of immunocompromised individuals. The treatment of fungal infections is challenging given the similarities of potential targets in the human host, the requirement for prolonged treatment regimens, and a limited selection of clinically effective, nontoxic antifungal therapeutic options.
Methods: Our research program aims to define how a fungal pathogen interacts with the host and understand why the host is unable to clear infection. Focusing on Cryptococcus neoformans, a highly-prevalent fungal pathogen among immunocompromised individuals, we exploit our extensive quantitative proteomics datasets of the interaction between host and pathogen at the protein level to assess options for reducing fungal virulence and combatting infection.
Results: We provide new insights into how fungi cause disease and the mechanisms used to evade the immune response. We also identify new strategies to perturb the interaction between pathogen and host to reduce our reliance on current antifungals for treatment options.
Conclusions: This information will support the reduction of selective pressure against antifungal-resistant strains and provide new tools against emerging resistant pathogens.

P09.13

Proteomic Unraveling of the Hidden Regulators of Erythropoiesis

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Introduction: The production of red blood cells (RBCs), termed erythropoiesis, is a hierarchical process starting from multipotent hematopoietic stem cells that through intermediate stages commit to erythroid-restricted progenitor cells, expand, and finally mature to RBCs. Importantly, the commitment, expansion, and maturation stages take place in different micro-environmental “niches”. The “erythroid expansion” niche is poorly defined to date. During fetal development as well as severe anemia, the spleen and liver are used as effective locations to expand the erythroid progenitor population. However, relatively little is known about the micro-environment and its mechanistic regulation of erythroid progenitor expansion, particularly under anemic conditions. We hypothesize that the regulators in the erythroid expansion niche are either cell surface proteins of neighboring cells, locally secreted, or proteins circulating in the extracellular space. Here, we have compared the plasma as well as splenic tissue interstitial fluid (TIF) proteome of healthy and anemic mice by high-resolution ion-mobility data-independent acquisition (FAIMS-DIA) mass spectrometry.
Methods: Spleen TIF and plasma from peripheral blood were collected from control and anemic (phenylhydrazine treated) mice in three biological replicates. Peptides from spleen TIF and plasma were extracted with an optimized sample preparation protocol and acquired in DIA mode with compensation voltage (CV) switching, on the Thermo Orbitrap Exploris 480 MS with FAIMSPro interface coupled to UltiMate 3000 RSLCnano. DDA runs were performed using HpH-RP fractionation. Spectral libraries generation and DIA data analysis were performed in Spectronaut (Biognosys).
Results: To understand the regulators of erythropoietic expansion, we analyzed the spleen TIF and plasma with multi-CV FAIMS-DIA. The spectral libraries with FAIMS annotation allow for deeper proteome quantitation and identified novel erythroid regulators.
Conclusions: Our approach provided a comprehensive overview on the proteome distribution in mouse plasma and spleen interstitial fluid upon anemic stress. Thus, it allows for deeper insights into regulation of erythropoiesis.

P09.14

Effects of Salicornia-Based Cream Skin Application on a Human Experimental Model of Pain and Itch.

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Introduction: Halophyte plants are salt-tolerant and are acclimated for growth in saline soils such as along coastal areas. Different types of halophytes have been used as both folk medicine and functional feed for multiple years, and an example is a species Salicornia of the subfamily Chenopodiaceae. The anti-inflammatory effects and antioxidative effects of Salicornia have been largely investigated, but the anti-inflammatory mechanisms and effects of the second metabolites contained in this plant for pain and itch sensation are still unclear.
Methods: Four forearm skin areas in 30 healthy volunteers were treated with 10% Salicornia-cream and vehicle cream for 24 or 48-hour. At day 0 skin permeability, thermal detection pain thresholds, mechanical pain thresholds and sensitivity, assessment of neurogenic inflammation, mechanical evoked itch were performed as baseline test. After 24 or 48-hour after creams application all test were re-assessed and application of 1% histamine and cowhage spicules was performed to evaluate the effects of Salicornia-cream on pain and itch skin reduction. Proteomic analysis of in-vitro Salicornia-extract treated fibroblasts by quantitative proteome analysis using DIA-PASEF is on-going.
Results: In this study skin permeability was reduced in Salicornia 48-hour treated areas compared to 24-hour treated areas($P<0.05$). Participants reported in Salicornia 48-hour treated areas a decrease in mechanical evoked itch sensation compared to participants treated for 24 hours($P<0.05$). Histamine induced neurogenic inflammation showed a significant reduction in 48-hour Salicornia-treated areas compared to 24-hour Salicornia treatment ($P<0.05$).
Conclusions: In conclusion 48-hour Salicornia treatment showed a significant effect on skin barrier architecture and reduced the reported mechanical evoked itch sensation from healthy volunteers. Likewise, neurogenic inflammation was reduced in the area treated with Salicornia for 48 hours, although no effects were present for pain reduction. Proteomic analysis of in vitro treated fibroblasts will help elucidate the mode of action of Salicornia extract on skin cellular components.

P09.15

Transcriptional and Translational Dynamics Underlie Synergy in Endothelial Inflammation

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Introduction
Vascular endothelial cells (ECs) form a dynamic interface between blood and tissue mediating critical steps in the immune response. Endothelial dysfunction through deregulation of cytokine stimuli underlies several vascular inflammatory disorders. However, the endothelial response to many cytokines is not known clouding target discovery to combat endothelial dysfunction.

Methods
To generate an overview of the molecular response of ECs to cytokines, we evaluated changes in the proteome of BOECs (Blood Outgrowth Endothelial Cells) stimulated to a panel of 92 cytokines separately and in combination. We then performed a time-resolved multi-omics analysis on BOECs exposed to TNFα and IFNγ, integrating transcriptome, whole (phospho-) proteome, and secretome.

Results
Out of 92 cytokines 47 had an impact on the endothelial proteome, ranging from broad proteomic responses to confined events. Co-expression-based clusters categorized 5 unique responses. TNFα and IFNγ induced distinct signatures on all omic levels, starting with phospho-events (mins), RNA regulation (>4h), followed by proteome (>8h) and secretome changes. Network analysis highlighted central hubs correlating to TNFα activation of the NFKB pathway and IFNγ signaling through the JAK/STAT pathway. Other IFNγ upregulated hubs included MHC-I/II complexes, secretion of T-cell attractants and complement factors. Combined TNFα and IFNγ stimulation increased molecular events on all omics levels. Interestingly, several mRNAs, such as transcription factor RELA, were only induced through combined stimulation. Moreover, a subset of mRNAs upregulated through a single stimulus, only increased on protein level after combining both stimuli, among which chemokines CCL5 and CXCL10.

Conclusions
Our integrated analysis highlights the intricate architecture of the endothelial response to different cytokines. The synergistic dynamic between transcript potentiation by an initial stimulus, and translation through a secondary inflammatory impulse suggests an integrated translation control which could underlie two-hit vascular inflammatory models. Exposing the regulation of this unresolved inflammatory priming could uncover new therapeutic avenues.

P09.16

Comprehensive Biological Characterization of Novel Antitumor Nanoconjugates by Newly Synthesized Proteomes with Bioorthogonal Non-canonical Amino-Acid Tagging.

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Introduction: The overwhelming size and complexity of human proteome require very high-throughput techniques for deep and rapid analysis; in this work, it is systematically characterized novel multifunctional nanoparticles (NPs) by high-content quantitative proteomics strategies to decipher detailed insights related to the interactions with the environment and intracellular pathways in different tumor cell lines.
Methodology: Here, a technique based on protein labeling with non-canonical amino acid azidohomoalanine (AHA) provided the ability to specifically and selectively detected newly synthesized proteins uniquely related to intracellular pathways triggered by novel NPs. Tumoral cells lines were exposed to the functionalized NPs including in the cell culture medium AHA amino acid that was integrated into the nascent proteins. Further bio-orthogonal chemistry between the amino acid and a copper-catalyzed azide-alkyne ligation allowed isolation of tagged proteins
Results: Attending to the total number of proteins, more than 1500 proteins were detected in each condition, obtaining more than 500 intracellular pathways by functional enrichment analysis (FEA). The FEA was simplified into broader functional groups according to Lin's semantic using the REVIGO method and provided a complete insight into the pharmacological characteristics associated with the NP and its different functionalities
Conclusion: Complete description of the environmental, drug delivery and biological activity of novel NPs has been depicted thanks to the high-throughput protein labeling technique.
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P09.17

Proteomic Basis for Understanding the Combination of Gemcitabine and Kinase Inhibitors to Kill Pancreatic Cancer Cells

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with limited treatment options. Most commonly, the DNA-damaging agent gemcitabine is used as first-line chemotherapy but chemo-resistance is frequently observed. One potential way to overcome gemcitabine resistance is the combination with molecularly targeted agents such as kinase inhibitors. This project aims to use proteomics to characterize the combination of gemcitabine and kinase inhibitors in PDAC cells.
Methods: High-throughput sensitivity profiling of 16 human PDAC cell lines towards >140 kinase inhibitors in combination with gemcitabine was performed. For proteome analysis of PDAC cell lines, samples were fractionated into 48 fractions and measured on a microflow-LC-MS/MS system as previously described (1). For phosphoproteome analysis, phosphorylated peptides were enriched from pooled fractions using IMAC and analysed by nano-LC-MS/MS. Based on the phenotypic screen, the ATR inhibitor (ATRi) elimusertib was selected for chemoproteomic target profiling using the Kinobeads technology. Therefore, the ATRi was allowed to bind kinases from AsPC-1 cell lysate in a dose-dependent fashion, followed by kinase-enrichment and LC/MS-MS analysis.
Results: Of all tested drugs, phenotypic combination screening identified ATRi elimusertib to synergize most effectively with gemcitabine in PDAC cell lines. Proteomic target profiling revealed high selectivity of elimusertib towards ATR, indicating that the cytotoxic effect upon combination with gemcitabine indeed comes from ATR kinase engagement and not from off-target effects. Deep proteome profiling of 16 PDAC cell lines defined the protein expression and phosphorylation levels for several thousand proteins, which was used to explain the observed phenotypic responses.
Conclusions: In conclusion, this project shows that integration of phenotypic and proteomics data can elucidate the mode of action of kinase inhibitors. This knowledge may further help to rationalize the use of kinase inhibitors in gemcitabine-based combination therapies in pancreatic cancer.

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P09.18

Developing and Validating a Set of Targeted Mass Spectrometry Assays for Pan-Herpesvirus Viral Protein Detection and Monitoring of Infection Progression

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INTRODUCTION: The presence and abundance of viral proteins within host cells signify the cellular stages of viral infections. Viral proteins are either brought into cells by infectious particles or expressed at specific steps of the replication cycle. However, methods that can comprehensively detect and quantify these proteins are limited, particularly for viruses like herpesviruses that boast large protein coding capacities. Importantly, the development of such methods would provide a comprehensive portrait of viral replication and allow for the screening of small molecules and other cellular perturbations with potential therapeutic and clinical applications.

METHODS: By integrating mass spectrometry and molecular virology, we have designed and experimentally validated a set of targeted proteomics assays for monitoring human viruses representing the three Herpesviridae subfamilies—herpes simplex virus type 1, human cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus. Our assays target hundreds of peptides covering 50-80% of the predicted proteomes for each of these viruses and span proteins that are representative of different temporal protein expression classes as well as virion components.

RESULTS: During wild-type virus infections, we first demonstrated that the breadth of proteins monitored by our methods captures the temporal cascades of the replication cycles of these viruses. Additionally, we illustrated that our assays can detect viral proteins at clinically relevant levels of infection. We next showed that these assays can be used to quantify the effects of long-established and recently-discovered antiviral agents, and further captures their precise temporal regulation of specific viral proteins. Finally, we demonstrated their broad utility for monitoring different viral strains, including laboratory and clinical isolates.

CONCLUSIONS: Altogether, our assays provide a reproducible framework for monitoring the progression of herpesvirus infections and are broadly applicable across a variety of model systems and contexts, including drug screening, detecting infections in clinical settings, and genetic manipulations of virus or host factors.

P09.19

Target Identification of a Natural Compound Regulating Mitochondria-ER Interaction Using DARTS-LC-MS/MS

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Traditional medicinal plants have been widely used as pharmaceutical agents due to their fascinating biological activities in vivo. Among them, we recently identified a natural plant Danshen is effective in ameliorating atherosclerotic plaque formation on ApoE-/- mice. Compound YCGX, an active principle of Danshen, was previously known to inhibit angiogenesis and induce autophagy in cancer cell lines but the underlying mechanism for autophagy in endothelial cells remains to be uncovered. In this study, we revealed that compound YCGX reduces the mitochondria-ER contacts leading to induce autophagy in a time dependent manner in HUVECs. To investigate the mode of action of compound YCGX, a combination of drug affinity responsive target stability (DARTS) and LC-MS/MS method was applied to identify the target protein of compound YCGX. Proteomes were analyzed by the averaged quantitative SWATH analysis and target protein of the compound was validated by cellular thermal shift assay (CETSA). We found that YCGX binds to an endoplasmic reticulum (ER) chaperone protein belonging to the GRP family. In addition, interaction of the compound with an ER protein was validated with knockdown of the target gene resulting in increase of the autophagic activities. Collectively, this study provides new insights into the mechanism of an anti-atherosclerotic natural compound in targeting mitochondria-ER contact complex for autophagy induction.

P09.20

The Cell Membrane Proteome: From Cancer Hallmarks to Therapeutic Interventions

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Introduction: The remarkable ability of cancer cells to enact aberrant proliferation programs and metastasize to distant sites is mediated via an altered cell surface proteome that facilitates in-and-out cell signaling processes as well as adhesion and migratory functions. In this work, we aimed at characterizing the cell membrane proteome of HER2+ breast cancer cells to identify proteins that synergistically contribute to uncontrolled cell proliferation, and that represent promising drug targets for effective networked therapeutic interventions with less side effects and toxicity.

Methods: Cell membrane proteins were isolated by the biotinylation of amino and glycan groups followed by affinity streptavidin pulldown, as well as by tryptic shaving of receptors in cell culture. Proteolytic digests of the isolated proteins were analyzed by nano-LC/QE-Orbitrap-MS, and raw data were processed by ProteomeDiscoverer 2.4. Proteins matched by two unique peptides with FDR<3 % were selected for bioinformatic analysis.

Results: As many as ~1300 cell membrane proteins could be matched to multiple hallmark categories with cell communication/signaling, adhesion/motility, immune response and cell cycle/growth accruing the largest number of protein hits. The genes corresponding to these proteins were spread over the entire genome, except chromosome Y. Altogether, the enrichment process enabled the classification of about 275 proteins with catalytic and receptor activity, 89 CD antigens, 255 cell adhesion/junction molecules, and 279 transport proteins. Among these, ~50 proteins already had approved, investigational, or experimental cancer drug targeting data. Of particular interest was the presence of antigen immunological markers that define the epithelial, mesenchymal or stemness characteristics of cells, and of cancer cell receptors that trigger cytotoxic innate and adaptive immune system responses that are key to helping cancer cells evade immune destruction.

Conclusions: The complex landscape of the cancer cell membrane proteome points to unique opportunities that can be exploited to guide immuno-oncology and precision medicine therapeutic approaches.

P09.21

The Chemoproteomic Target Landscape of HDAC Inhibitors Highlights MBLAC2 as Common Off-Target

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Introduction: Drugs targeting class I histone deacetylases (HDACs) are approved for several oncological indications. For neurological disorders, isoform-specific inhibitors of class II HDACs are investigated but need to be selective to avoid off-target-mediated toxicity. Defining the target landscape of HDAC drugs is therefore central to drug development. Importantly, the embedding of HDACs in large complexes influences drug affinity but is not considered in classical recombinant enzyme activity assays.

Methods: We prepared novel affinity probes to enrich human HDACs. Based on those we optimised a chemoproteomic competition assay and profiled 53 drugs (9 doses response) for selectivity. Recombinant MBLAC2 activity assay, siRNA knockdown, lipidomics and extracellular vesicle counting were used to support MBLAC2 annotation.

Results: With an optimised chemoproteomic competition assay featuring three promiscuous probes, we established the selectivity profiles of 53 drugs(1). The obtained target landscape spans 9 of 11 Zinc-dependent HDACs and 5 other proteins, including the ill-annotated metallo-beta-lactamase-domain containing protein 2 (MBLAC2). Our results recapitulate known trends of drug selectivities but also question the reported selectivity of widely-used molecules. We propose alternative probe-candidates with exceptional selectivity for HDAC6/10. The co-competition of class I HDAC complex members, allowed to probe endogenous HDAC complexes: we notably found an over ten-fold difference in drug binding affinity for HDAC1 when part of a RCOR1- compared to RCOR3- containing CoREST complex. Unexpectedly, our landscape established MBLAC2 as a frequent hydroxamate drug off-target. Accumulation of extracellular vesicles was found to be an intriguing phenotype of MBLAC2 inhibition or knockdown.

Conclusions: Systematic drug profiling using chemoproteomics helps expand the ligandable proteome. Many hydroxamate molecules, including clinically advanced drugs, were indeed surprisingly found to inhibit MBLAC2. We demonstrated the involvement of this hydrolase in extracellular vesicle biology, creating an incentive for drug discovery in e.g. immunology and oncology, where exosomes play an important role.

(1)Reference: https://www.researchsquare.com/article/rs-646613/v1

P09.22

Label-Free Dia-PASEF Compared to TMT Quantitation for Thermal Proteome Profiling / Cellular Thermal Shift Assay

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Introduction
Cellular thermal shift assay (CETSA) coupled with high-resolution mass-spectrometry (MS) has proven to be indispensable tool to track thermal stability changes in cellular proteins caused by various external or internal perturbations (10.1126/science.1255784). Recent advances in data-independent acquisition (DIA) and gas-phase peptide separation (PASEF) significantly increased sensitivity and throughput in label-free protein quantification. Here dia-PASEF was evaluated in comparison to higher degree multiplexed data acquisition (doi:10.1038/s41592-020-0781-4) for profiling Staurosporine, a thermal stability profiling "gold-standard" (doi:10.1101/2020.03.13.990606).
Methods
For the experiment, clarified K562 lysate was incubated with either 20µM of Staurosporine or vehicle control for 15 minutes. After CETSA heat shock, sample were pooled (compressed) and aggregated proteins were removed by centrifugation. Sample processing used standard methodology. NanoLC-MS data was collected on a timsTOF Pro - nanoElute set-up using 30 min (spectral library, high-PH RP fractions; 400 ng dia-PASEF), 60 min (800 ng dia-PASEF) or 100 min format (spectral library, 800 ng dda-PASEF). Data analysis used Spectronaut 14 with addition of PEAKS+ for dda-PASEF data.

Results
Traditional spectral library generation (Pulsar search, 72 fractions) identified 8135 protein groups based on almost 100 K unique peptides. For the dda-PASEF approach (100 min gradient, 800 ng), Pulsar and PEAKS+ search identified 5103 and 4723 protein groups based on 76753 and 72247 peptide sequences, respectively. Combined, the results yielded a hybrid library of 5400 protein groups and 69000 peptides. Statistical analysis of the data resulted in identification of 72 proteins with significant Staurosporine induced-thermal stability changes, majority of them annotated as kinases.

Conclusions
Application of dia-PASEF data collection for CETSA MS opens possibility for increased throughput of thermal proteome profiling, as well as reducing reagent costs and processing time.
These results are similar to previously published TMT-based results.

P09.23

Proteomics Profiling of Systemic Effects of Bovine Colostrum Diet in Preterm Piglets - A Translational Model for Neonate Disease Pathology

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Introduction: Nutrition plays a significant role in whether preterm infants (born <37 weeks of gestation) receive a good beginning at life. Unfortunately, mother's own milk is often short in supply following preterm birth, and donor milk and infant formula are the alternatives. Yet, donor milk is not always available and infant formula has been associated with increased risk of necrotizing enterocolitis (NEC). Bovine colostrum as a diet in preterm piglets has shown to protect against NEC; thus, we aim to investigate the systemic proteome differences between bovine colostrum and infant formula fed preterm piglets using quantitative proteome analysis.

Methods: 25 preterm piglets (delivered at 90% gestation) were fed with infant formula (n=13) or bovine colostrum (n=12) for 9 consecutive days. Plasma samples from day 5 and day 9 were used for label-free quantitative diaPASEF based LC-MS/MS analysis.

Results: We identified 564 proteins, from which 28 and 9 proteins were differentially expressed on day 5 and day 9, respectively. Functional enrichment analysis revealed downregulation of proteins involved in regulation of acute inflammatory response in bovine colostrum fed piglets on day 5, whilst upregulated proteins were involved in developmental processes, including skeletal system development. Meanwhile, the 9 regulated proteins on day 9 were all downregulated in bovine colostrum fed piglets, and were involved in platelet degranulation, regulated exocytosis, and cell adhesion.

Conclusion: The differentially expressed proteins suggest that the biggest effect between the diets is observed early on, whilst the difference in proteomes becomes smaller on day 9. Furthermore, the upregulated proteins in bovine colostrum fed piglets seem to be associated with developmental pathways, indicating better growth in these piglets.

P09.24

Target Identification, Selectivity Profiling and Mechanistic Insights of a Cdk9 Inhibitor Using Complementary Proteomics Methods

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Introduction: In an effort to map the selectivity and understand the mode of action of a Cdk9 inhibitor (Compound 1), we used several orthogonal proteomics methods. Here we describe the methods used, their strengths and weaknesses, how they can be used in the drug discovery pipeline and how they synergize to provide mechanistic insights of compounds of interest.
Methods: We have used Chemoproteomics, kinase affinity tools, Cellular Thermal Shift Assay (CETSA) and Limited Proteolysis (LiP). These techniques all rely on LC-MS/MS based analytical platforms, but place different demands on the sample or require different sample handling protocols and subsequently also have different strengths and weaknesses for target deconvolution
Results: The results obtained clearly confirm cdk9 as the primary target of Compound 1, with affinity curves highly correlated between the different target deconvolution techniques.
The chemoproteomic approach rely on derivatized compound, tethered to a sepharose bead. Subsequently, the binding competition assays are performed on lysed cell material. The choice of a mild lysis buffer allowed us to identify, not only CDK9, but also it's molecular partners in the p-Tefb complex with similar concentration response behavior. The results for the kinases identified in the study were strikingly similar also without the chemical modification using the kinobead assay as well as the lysate based CETSA experiments. In the intact cell version of CETSA, not only the direct binders of the compound show stability shifts, but also downstream events and other secondary modulatory effects leave thermal traces in the cell, shedding light on the cellular response to the compound. Finally, Limited Proteolysis was used to identify target proteins and allowed mapping of peptides directly adjacent to the ATP binding pocket of CDK9.
Conclusion: The use of complementary techniques, based on unique biological and biochemical processes, allow robust and confident characterization of protein inhibitors.

P09.25

Systems-based Examination of DLK-MAPK Signaling in Human Stem Cell Derived Retinal Ganglion Cells during Cell Death

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Glaucoma is characterized by progressive loss of retinal ganglion cells (RGCs) and while reasonable therapies exist – primarily to lower intraocular pressure – they are not always effective. To date, there are no FDA-approved neuroprotective agents that directly halt vision loss. Our goal is to increase our understanding of RGC neurodegenerative and neuroprotective signaling networks during axonal injury using global and phosphoproteomics and proximity labeling interactomics in human stem cell-derived RGCs (hscRGCs), a highly disease-relevant model system. The dual leucine zipper kinase (DLK)/leucine zipper kinase (LZK)/c-Jun N-terminal kinase (JNK)/mitogen activated protein kinase (MAPK) pathway initiates apoptosis in response to axonal injury and blocking DLK activity is protective to RGCs in various models, including hscRGCs. Thus, our primary focus is on the signaling events within the DLK-LZK-JNK-MAPK axis that cause cell death upon axonal injury, as well as what acts upstream to activate this toxic signaling cascade.
To this end, we measured early hscRGC proteome and phosphoproteome perturbations caused by axon injury using the microtubule destabilizing agent colchicine and how these perturbations are affected by neuroprotection using DLK/LZK-targeting kinase inhibitors. In parallel, we surveyed the DLK interactome in hscRGCs expressing a BioID2-DLK fusion protein under normal conditions and colchicine treatment.
We identified and quantified more than 9,000 proteins and 12,000 phosphosites in these cells and revealed nearly 200 candidate phosphosites and proteins for DLK-mediated cell death effectors. Interactome analysis revealed roughly 80 potential interaction partners in these cells, with several candidates overlapping in both experiments and others standing out as candidates for DLK activation. Together, our analyses suggest a future focus on the intermediate filament system, protein translation, and unexplored members of the MAPK pathway.

P09.27

Establishment and Characterization of a Novel Cancer Stem Cell Derived from Cholangiocarcinoma by Proteomics

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Introduction: Cholangiocarcinoma(CCA) is an aggressive and highly metastatic with poor response to chemotherapy and high recurrence rate. Cancer stem cells(CSCs) have been demonstrated to contribute to chemotherapy-resistances and aggressive phenotype/recurrences of many cancers including CCA. Therefore, CSCs have been proposed as an effective targets of cancer. However, the study of CSC in CCA is very limited since there are few CSC model cells and their molecular markers.
Method: We established CCA-stem-like cells from CCA, (KKU-055-CSC), under the specific condition of stem cell medium. Differentiation(DIF) of CSC to cancer cells is induced by the 10%FCS. The characterization of CSC has been performed by Western-Blotting(WB), ICC, cellular proliferation/drug-resistance assay and mouse transplantation. The proteomics of those cells were performed by the LFQ using an Easy-nanoLC-Orbitrap-Fusion-Tribrid system, equipped with Nikkyo-C18-nano-Column, The data normalization/mining/statistics was performed by Proteome Discoverer(ver2.4)/MaxQuant/Perseus software. GO analyses and network analyses were assisted by DAVID/KEGG/KeyMolnet.
Results: We successfully established and characterized KKU-055-CSC which has a potential to form malignant tumor in mouse model. KKU-055-CSC expresses the stem cell markers such as SOX2/CD44/OCT3/4, shows the drug resistance against 5FU/cisplatin/gemcitabine, and possesses the activity of multi-lineage-differentiation. To understand their signaling pathways controlling KKU-055-CSC stemness, we performed comparative proteomics between stem cells(KKU-055-CSC), differentiation induced(KKU-055-DIF) and cancer(KKU-055-Pareantal) cells. The global proteomics identified 7,048 proteins in total, followed by the cluster extraction, 202 molecules up-regulated in KKU-055-CSC (>1.5-fold, p-value <0.05) and down-regulated in both KKU-055-DIF and KKU-055-Pareantal were identified. The network analysis revealed that the signaling pathways of Aurora, transcriptional regulation pathway by high-mobility-group-proteins(HMGP) and CD44 were significantly upregulated in KKU-055-CSC. The validation with WB/ICC confirmed the specific upregulation of these proteins in CSCs.
Conclusion: We established a novel CCA-CSC and identified the Aurora-HMGP-CD44 signaling pathway in CSC by proteomics, which may be a specific candidate of therapeutic marker and target against CSC in cholangiocarcinoma.

P09.28

Dynamic Polygon for MHC Class I and II Immunopeptides

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Introduction: MHC-associated peptides powerfully modulate T cell immunity and play a critical role in generating anti-tumor immune responses. Characterization of these peptides helps to generate therapeutic treatments. These peptides are challenging to characterize due to similar length and lacking defined termini compared to tryptic peptides. To overcome these challenges, use of PASEF enables to generate high quality peptide spectra and resolve coeluting and isobaric peptides. Moreover, the capability to easily tailor the mobility space enables preferential detection of groups and sub-groups of relevant peptides.

Methods: MHC I peptides were separated on a 100 minutes gradient by nanoElute UPLC on a 25 cm column and analyzed on a high resolution TIMS-QTOF. For MHC II peptides separation was performed on an EvoSep system (60 samples per day method) and analyzed on the same instrument. Data analysis was performed with PMI Byonic.

Results: The unsurpassed speed of the timsTOF Pro enable detection of 16,000 peptides and 4,000 protein groups (PG) on average for each one of the 3 replicates for the MHC I sample (200 ng on column). Several isobaric and isomeric peptides, yet with completely different sequences, but overlapping retention time are distinctively separated by IMS and subsequently fragmented without generating chimeric spectra. Two different polygons were utilized for scouting purposes. A broad polygon that included 1+ ions generated approximately 20% more peptide IDs and 5% more PG. For MHC II sample, with EvoSep platform (25 ng injected) more than 2000 peptides and 500 PG were detected.

Conclusion: Distribution of N-mer for MHC class I shows peptides with 9 amino acid residues, by far as the most abundant analytes. For MHC class II peptides, N-mer varying from 14 to16 amino acidic residues are detected as being the most abundant ones. These trends match what has been reported in the literature.

P09.29

Metalloproteomic Analysis of Brazilian Snake Venoms as Proof of Concept for the Development of a Diagnostic Kit for Snakebites

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Introduction: Snakebite envenomation is known to be a neglected tropical disease that kills more than 100,000 people and causes sequelae in more than 400,000 people every year worldwide. According to epidemiological data on humans, more than 110,000 Brazilians are affected by accidents by snakes annually, and of these, 900 Brazilians are left with sequelae and 125 die on average every year. It is believed that the rapid diagnosis about what snake caused the accident by the health agents would be of great importance for the correct and immediate administration of the specific antivenom and adjuvant treatments. Consequently, these devices would avoid the aggravation of the clinical condition of the patient, given the delay in identifying the type of snake that affected the patient. So, the objective of the present work was to evaluate the composition of metallic ions present in the venom of Brazillian healthy snakes and to correlate with the abundance of metalloproteases (SVMPs).
Methods: Snakes venoms were subjected to shotgun proteomics strategy by using Q-Exactive mass spectrometry and the analysis of Mg, Zn, Ca, Fe, Mn, Cu metals ions by atomic spectrometry.
Results: Proteomics analysis have evidenced less than 1% of SVMPs in snakes of the genus Crotalus, 15% of SVMP in Bothrops spp and 25% in Lachesis spp. Mg ions can indicate envenomation by Crotalus genus snakes, while Cu ions can indicate envenomation by snakes of the Lachesis genus. Zn ions can be useful for the detection of envenoming by snakes of the Bothrops genus.
Conclusions: The metalloproteomic profile of main Brazilian snake venoms in Brazil were evaluated and the results support strategies for the development of a rapid diagnostic kit to detect which animal caused the snakebite accident.

P09.30

Quantitative Proteomics Shows High Selectivity and Reveals the Mechanism-of-Action of a STAT3 Degrader

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Introduction:
Signal Transducer and Activator of Transcription 3 (STAT3) is a transcription factor which plays important roles in regulating many biological processes. Aberrant STAT3 activation is associated with many serious diseases including various lymphomas and leukemias. Regulation of STAT3 activity has eluded traditional drug design approaches and has long been considered an “undruggable” target. In the past decade, targeted protein degradation has emerged as a novel therapeutic modality which modulates disease biology by harnessing the cellular ubiquitin proteasome to selectively degrade proteins. KTX-201 is a heterobifunctional targeted protein degrader of STAT3 being used as a tool for the treatment of hematologic malignancies.
Methods:
To investigate its selectivity and mechanism-of-action, SU-DH-L1 anaplastic large cell lymphoma cells were treated with KTX-201 between 8 and 48 h in duplicates alongside DMSO controls. Tryptic peptides were labeled with TMTpro 16-plex reagents, subjected to high pH fractionation and analyzed using an Orbitrap-Eclipse mass spectrometer.
Results:
In total, nearly 9,300 proteins were quantified, including STAT3 and other STAT family members. More than 90% degradation of STAT3 was observed across all four time points. At 8h, STAT3 was the only significantly changed protein, demonstrating a high level of selectivity of the degrader. At 16h and 24h, a small group of proteins involved in the cytokine-mediated signaling pathway were significantly downregulated in response to STAT3 depletion, including SOCS3, MYC, IL-2RA, CEBPB and GZMB. The most profound proteome changes were observed at 48h. In addition to the cytokine-mediated signaling pathway, many significantly down-regulated proteins were related to cell cycle and mitotic regulation, indicating the potential mechanism for the anti-tumor activity of KTX-201.
Conclusions:
In summary, this study demonstrates that KTX-201 is a highly selective degrader of STAT3, a target traditionally considered “undruggable, and further serves as a starting point for the selection of proof of mechanism biomarkers in clinic.

P09.32

Integrated Proteomics Revealed Acetylation-Induced PCK Isoenzyme Transition Promotes Metabolic Adaptation of Liver Cancer to Systemic Therapy

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Introduction Sorafenib and lenvatinib are approved first-line targeted therapies for advanced liver cancer, but most patients develop acquired resistance. Except for primary resistance, most patients develop acquired resistance within 6 months. Unfortunately, due to the complex pharmacological effect of sorafenib, there is currently no available biomarker to predict the therapeutic response to sorafenib in the clinic. Methods Given that sorafenib is a multiple tyrosine kinase inhibitor, we subjected liver cancer cell HepG2 (HepG2-WT) and sorafenib-resistant HepG2 (HepG2-R) cells to integrated proteomics, phosphoproteomics and acetylproteomics analyses to elucidate the mechanism of metabolic reprogramming in sorafenib-resistant cells. Results Quantitative proteomics analysis identified a total of 4984 proteins, with 4365 proteins having a 1% false discovery rate at both the peptide and protein levels. Phosphoproteomics analysis identified a total of 7459 phosphosites corresponding to 2508 phosphoproteins. A total of 350 and 136 phosphosites mapping to 275 and 124 phosphoproteins was identified as being increased and decreased. Acetylproteomics analysis revealed a total of 895 acetylated sites, corresponding to 543 acetylated proteins. We found that sorafenib induced extensive acetylation changes towards a more energetic metabolic phenotype. Metabolic adaptation was mediated via acetylation of the Lys-491 (K491) residue of phosphoenolpyruvate carboxykinase isoform 2 (PCK2) (PCK2-K491) and Lys-473 (K473) residue of PCK1 (PCK1-K473) by the lysine acetyltransferase 8 (KAT8), resulting in isoenzyme transition from cytoplasmic PCK1 to mitochondrial PCK2. KAT8-catalyzed PCK2 acetylation at K491 impeded lysosomal degradation to increase the level of PCK2 in resistant cells. PCK2 inhibition in sorafenib-resistant cells significantly reversed drug resistance in vitro and in vivo. High levels of PCK2 predicted a shorter progression-free survival time in patients who received sorafenib treatment. Conclusions Acetylation-induced isoenzyme transition from PCK1 to PCK2 contributes to resistance to systemic therapeutic drugs in liver cancer. PCK2 may be an emerging target for delaying tumor recurrence.

P10.01

Global Proteome Expression Study of Patient-Derived Sarcoma Cell-Lines toward Optimization of Therapeutic Strategy Using Relocated Anti-cancer Drug

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[Introduction]
Sarcomas are malignant tumors of mesenchymal tissues, and rare that the incidence is estimated to be 50 cases per 1 million population. The prognosis of sarcomas is dire, and the gold-standard therapy is not yet established.
In previous studies, we discovered a certain drug (Drug X) that was effective to sarcomas by screening of already pre-existing anti-cancer agents using 35 types of sarcoma-derived cell-lines. The purpose of this study is to explore proteins that are related to the effectiveness of Drug X to optimize the therapeutic strategy.
[Method]
We exploited the 35 cell-lines which were used in previous studies, and classified them into two groups according to the response to Drug X. Mass spectrometric protein expression profiling was performed in all cell-lines, and we explored the proteins whose amount showed the statistically significant difference between the groups. We utilized student-t analysis in each protein, and set the p value to 0.05 as statistical significance.
[Result]
We generated mass spectrum protein expression profiles of all 35 cell lines examined in this study. The identification of intriguing proteins will lead innovative seeds which will allow optimization of the relocated drugs.
[Discussion]
The experiments using patients derived cell-lines are competent not only for pre-existing drug relocation, but also for searching the protein that may be associated with drug effect. This study gives potent clues to elucidate the new predictive biomarker as well as a mechanism of response to anti-cancer agents.

P10.04

Cell Surface Phenotyping of the Human Heart Reveals Cardiomyocyte-Specific Targets and Surfaceome Dynamics of Explanted Cardiac Fibroblasts

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Introduction: In the heart, cell surface glycoproteins in cardiomyocytes and cardiac fibroblasts are essential for sustaining normal organ function and play critical roles in cardiac development, disease, and drug uptake. However, the lack of a detailed cell type- or chamber-resolved view of the cell surface proteome of the adult human heart currently limits discovery of new targets for precision drug delivery and the development of practical approaches for studying how different cell types contribute to the development of cardiac disease.

Methods: CellSurfer, a new analytical platform, was applied to cardiac cells isolated from human hearts. Briefly, cell surface N-glycoproteins on ~1 million cells were labeled, digested, selectively enriched using streptavidin magnetic beads, cleaned using SP2, and analyzed by MS. Sample preparation was automated using liquid handling robotics. MS data were analyzed using Proteome Discoverer, Spectronaut, MSstats, and R. Results were curated and annotated using Veneer.

Results: Integrating CellSurfer with an optimized strategy for isolating intact primary cardiomyocytes and fibroblasts from human donor heart tissue resulted in the generation of the first chamber-, cell-type-, and patient-specific map of the cell surface N-glycoproteome in the adult heart. Overall, >1200 cell surface N-glycoproteins were detected, including >100 cell surface proteins not previously described in these cell types. Novel monoclonal antibodies generated for one cardiomyocyte protein uniquely localize to cardiomyocytes within human heart tissue sections and stem cell derivatives, suggesting its value for cell-type specific targeting and immunophenotyping. Comparisons of explanted cardiac fibroblasts within the first three passages reveals previously undescribed remodeling of the surfaceome, justifying caution when using cultured cells.

Conclusions: These data represent the first major step towards a comprehensive, donor, cell-type, subtype, and chamber-resolved reference map of cell surface phenotypes in the adult human heart and reveal new targets for immunophenotyping, drug delivery, and benchmarking explanted cells and stem cell derivatives.

P10.05

The Secretome Deregulations in a Rat Model of Endotoxemic Shock

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Introduction. Septic shock is a systemic inflammatory response syndrome associated with organ failures. Earlier diagnosis would allow decreasing the mortality rate. However, there is currently a lack of predictive biomarkers. The secretome is the set of proteins secreted by a cell, a tissue or an organism at a given time and under certain conditions. The plasma secretome is easily accessible from biological fluids and represents a good opportunity to discover new biomarkers that can be studied with non-targeted "omic" strategies. Aims. To identify a relevant deregulated proteins (DEP) in the secretome of a rat endotoxemic shock model. Methods: Endotoxemic shock was induced in rats by injection of lipopolysaccharides (LPS, S. enterica typhi, 0.5 mg/kg, iv) and compared to controls (Ringer Lactate, iv). Under isoflurane anesthesia, carotid cannulation allowed mean arterial blood pressure (MAP), heart rate (HR) monitoring and blood sampling at different time points (T0, and 50 or 90 with EDTA and protease inhibitor). Samples were prepared for a large-scale tandem mass spectrometry (MS-MS) based on a label free quantification to allow identification of the proteins deregulated upon endotoxemic conditions. A gene ontology (GO) analysis defined several groups of biological processes (BP) in which the DEP were involved. Results: Ninety minutes after shock induction, the LPS group presents a reduction in MAP (-45%, $p<0.05$) and an increased lactate levels (+27.5%, $p<0.05$) compared to the control group. Proteomic analyses revealed 10 and 33 DEP in the LPS group respectively at 50 and 90 minutes after LPS injection. GO-BP showed alterations in response to oxidative stress and coagulation at 50 and 90 minutes respectively.
Conclusion. This study proposes an approach to identify relevant DEP in septic shock and brings new insights in the understanding of the secretome adaptations upon sepsis.

P10.06

Two Novel Serum Biomarkers Are Associated with the Serological Status of Rheumatoid Arthritis Patients: A Tool for Precision Medicine Strategies

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Introduction: Rheumatoid Factor (RF) and Anti-Citrullinated Protein Antibodies (ACPA) are key for the diagnosis of Rheumatoid Arthritis (RA). However, additional serological markers will improve the early diagnosis and treatment response of this disease, enabling further patient stratification and application of precision medicine strategies. In this work, we investigated serum proteins associated with RF and/or ACPA in RA patients.

Methods: An iTRAQ-based shotgun proteomic analysis was performed on sera from the RA cohort of the Hospital of Santiago de Compostela (CHUS). Sera were classified as seropositive or seronegative according to their RF and ACPA values. A Multiple Reaction Monitoring (MRM) method was developed using Skyline and the targeted analysis was performed using peptides with internal labelled standards. Serum levels of orosomucoid 1 (ORM1) and haptoglobin (HPT) were measured by ELISA in the RA cohort of the Hospital of A Coruña (HUAC).

Results: Eighty sera were grouped in 4 pools, according to their ACPA/RF status. LC-MS/MS analysis showed that the abundance of eleven proteins was altered in ACPApos/RFpos, 13 in ACPAneg/RFpos and 12 in ACPApos/RFneg, all compared to ACPAneg/RFneg. Vitamin D binding protein was the unique protein increased in all the comparisons. For verification, samples from the CHUS cohort were analyzed individually. Then, 26 peptides belonging to ten proteins associated with double positivity were quantified by MRM. Two acute phase reactants (ORM1 and HPT) were verified in this phase. The increase of these two biomarkers in the double seropositive status was then validated on 260 patients from CHUAC (p=0,0053 ORM1; p=0,0026 HPT). Finally, the increased level of ORM1 showed association with RF rather than ACPA (p=0,0008), whereas HPT with ACPA rather than RF (p=0,0112).

Conclusion: The determination of ORM1 and HPT in sera provides novel information useful for patient stratification, which might facilitate the development of personalized medicine in RA.

P10.08

Development of a Standardized MRM Targeted Proteomics Method for Monitoring One-Carbon Metabolism Enzymes in Hepatocellular Carcinoma and Cirrhosis

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Introduction. Liver cancer has an increasing incidence and represents one of the most frequent causes of cancer death worldwide, being the sixth most prevalent and the fourth with a highest lethality (8.2% cancer deaths. Hepatocellular carcinoma (HCC) is the predominant type of liver cancer and despite that most risk factors are known, HCC-associated mortality is still high due to late diagnosis. Therefore, there is a need for HCC biomarkers, especially in patients with cirrhosis, who are at higher risk of HCC development. In this regard, one carbon metabolism (1CM) arises as a promising pathway to explore liver physiology and stratify liver disease patients. We have demonstrated a deep reconfiguration of 1CM in mouse HCC showing different profiles associated to different aetiologies.
Methods. We have developed a targeted proteomics MRM method for robust detection and quantification of 13 enzymes involved in 1CM pathway in human liver. Sample preparation for MRM analysis consisted of tissue lysis and protein digestion with trypsin. Heavy synthetic peptides were spiked into each sample in a fixed and known amount in order to ensure accurate peak selection and quantification with Skyline. Standardization of the quantitative MRM method was done according to CPTAC guidelines.
Results. The performance of the method was tested in 63 human liver samples classified in 3 groups (cirrhosis, HCC and control). Statistical analysis was done according to light/heavy peptide ratio and revealed significant differences for GNMT, AHCY, CBS, MAT1A, MAT2A, BHMT, SHMT1 and SHMT2 between groups. Machine learning analysis revealed that the systematic quantification of 1CM enzymes allowed stratification of liver disease patiens with more than 80% accuracy.
Conclusion. The MRM method described here allow the robust and systematic quantification of 13 1CM pathway enzymes and might prove its value to classify liver tissue samples according the pathological condition of patients.

P10.09

The Proteomic Analysis of High Grade Serous Ovarian Cancer Reveals the Role of Tumor Microenvironment in Chemoresistance.

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Introduction:
High grade serous ovarian cancer (HGSOC), represents ~70% of epithelial ovarian cancer cases. Patients initially respond well to platinum-taxane combination chemotherapy, however ~80% relapse, and most experience chemo-resistance. Prior studies have profiled the genomic and proteomic landscape of ovarian cancer, but the role of tumor adjacent stroma and stromal infiltration in the context of ovarian cancer metastasis, recurrence and chemo-resistance is largely unknown.

Methods:
We performed proteomic analysis using label-free data-independent acquisition mass spectrometry (DIA-MS) in matched chemotherapy-naïve (primary) and resistant tumors (recurrent) and tumor adjacent stromal samples from 32 women. We used CAM3, a computational deconvolution tool to classify samples as tumor or stroma based on their protein expression profiles. We calculated stromal content of all samples using Hover-Net, a tool that simultaneously segments and classifies nuclei from histology images. Average stromal score was calculated from both CAM and Hover-Net.

Results:
We performed differential expression analysis (DEA) between stroma adjacent to primary or recurrent tumors. We identified 134 proteins upregulated in primary stroma and 51 proteins upregulated in recurrent stroma (FDR < 0.01, abs(log2FC) > 0.5). Proteins like S100A9 and LTF were upregulated in recurrent stroma and were significantly enriched in phosphoinositol signaling, differentiating T lymphocytes and hematopoietic stem cell pathways (GSEA, FDR < 0.05).
Regression analysis identified proteins positively correlated with an increasing percentage of stromal infiltration in tumor tissues (p-value < 0.05). These proteins were enriched in pathways like integrin cell surface interactions, VEGFA targets and KRAS targets (GSEA, FDR < 0.05). The top 10 positively correlated proteins included TAGLN2, Lum and AEBP1, which were previously implicated in cancer metastasis via the tumor microenvironment in colorectal, lung and breast cancers.

Conclusion:

Computational histologic and proteomic deconvolution informed DEA revealed novel stroma-specific drivers of chemoresistance in recurrent HGSOC.

P10.10

A Quantitative Discovery Platform to Survey the Human Blood Plasma Proteome in Precision Oncology

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INTRODUCTION
Mass spectrometry-based discovery proteomics has recently emerged as a high-throughput method for the proteomic profiling in biofluid samples from large clinical and population screening cohorts. Despite this progress, a significant fraction of the plasma proteome is currently not covered by state-of-the-art discovery approaches and therefore not accessible for biomarker discovery.
To close this analytical gap, we present a novel workflow combining automated plasma depletion and FAIMS-DIA-MS to bridge both sensitivity and scalability. We demonstrate the applicability of this workflow to support biomarker discovery and subject stratification in precision oncology in a case-control cohort.

METHODS
The plasma samples were depleted in 96-well format using an automated MARS-14 depletion system. The depleted samples were processed to tryptic peptides and analyzed using a Thermo Scientific Orbitrap Exploris 480 equipped with a FAIMS Pro device. Data processing and analysis were performed using Biognosys' SpectroMine and Spectronaut software.

RESULTS
Using the unbiased discovery workflow, we investigated a cohort comprising of 180 plasma samples from healthy donors and subjects diagnosed with pancreatic, breast, prostate, colorectal and lung (NSCLC) cancer at either early or late stage of the disease.
Overall, the optimized FAIMS-DIA-MS quantified 2,741 proteins across all samples and 1,849 proteins on average per sample measurement. Based on estimated plasma protein concentrations (Human Protein Atlas), quantified proteins span across 8 orders of magnitude, down to single digit pg/mL. Within this dynamic range, we could interrogate the tissue leakage proteome, interleukins and signaling proteins. Using classification algorithms, we were able to select candidates to build protein panels which provide significant positive predictive values associated with different disease stages, especially in the sub-cohorts for pancreatic and colorectal cancer.

CONCLUSIONS
We demonstrate the capabilities of a novel discovery workflow for deep, quantitative profiling of plasma samples at large scale, providing a rich proteomic resource for precision oncology.

P10.12

Functional Protein Discovery for the Early Diagnosis of Neonatal Sepsis

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Introduction: Sepsis is a frequent complication among newborns and accounts for >400,000 deaths globally. Infection-related inflammation contributes to long-term adverse neurodevelopmental outcomes in infants that survive sepsis. Preterm infants, particularly those born <32 weeks gestational age, are at the highest risk, affecting up to 22%, for developing sepsis. A rapid and accurate diagnosis of sepsis is critical to minimise inflammation and antibiotic therapy, but early diagnosis is complicated by slow (24-36 hours) and variable diagnostic tests. Consequently, 2/3 of infants receive unnecessary antibiotic therapy, which is associated with adverse outcomes, including mortality, and contributes to antibiotic resistant organisms in the community. Thus, there is an urgent and unmet need for accurate and more rapid adjunct diagnostics to reduce the high prevalence of antibiotic use in this vulnerable population. Proteome differences can be used to identify functional protein biomarkers that can improve the current diagnostic approach and potentially identify novel immune modulators for the prevention and treatment of sepsis.

Methods: Label-free quantitative diaPASEF LC-MS/MS with timsTOF PRO was used to explore the plasma proteome of human preterm infants born <29 weeks gestational age with and without sepsis (n=15 and n=39, respectively).

Results: In very preterm infants with and without sepsis, over 500 plasma proteins were identified. Over 70 differentially expressed proteins are associated with sepsis, 32 of which are up-regulated and 42 are down-regulated.

Conclusion: We identified known and novel proteins that are associated with sepsis. On-going analysis suggests that a subset of proteins may have clinical utility as biomarkers for early diagnosis of sepsis in very preterm infants.

P10.13

Fast Library Generation Using Zeno MS/MS and Microflow Chromatography

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Introduction: The generation of spectral libraries is often thought of as a time consuming procedure, but in reality they can be generated very quickly. Using microflow chromatography with very fast MS/MS acquisition on a QTOF system, then searching the data in the cloud can enable library generation in <1 day. Here this workflow was explored using the ZenoTOF 7600 system and the impact of larger libraries on DIA protein quantification was tested.
Methods: Here, a large number of fractions (40) from high pH reversed phase separations of complex digested samples (K562, Hela) were collected then analyzed using microflow chromatography (10 min gradients). Data dependent analysis was performed on the ZenoTOF 7600 system using Zeno MS/MS for fast, high sensitivity MS/MS analysis. Protein identification and SWATH acquisition data processing was performed in the cloud using OneOmics suite.
Results: The libraries generated from each fractionated cell line contained >8000 proteins and >175000 peptides. Comparing the proteins found from each cell line, >500 new proteins were added to the library by analyzing a second cell line. Processing SWATH acquisition data with the large, combined library enabled extraction of many more proteins from a standard cell line acquisition, resulting in a 30-40% improvement from the previously generated libraries on older platforms.
Conclusions: Using fast microflow LC and Zeno MS/MS combined with cloud-based data processing, very large scale libraries can be generated in under 24 hours. Higher quality MS/MS libraries can improve information content extracted for SWATH acquisition data.

P10.14

Precise Quantitation of PTEN by Immuno-MRM: A Tool to Resolve the Breast Cancer Biomarker Controversy

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Introduction
The tumor suppressor PTEN is the main negative regulator of PI3K/AKT/mTOR-signaling and is commonly found downregulated in many cancers such as breast cancer (BC). Conflicting immunohistochemistry (IHC) and western blot (WB) data have sparked a controversy about PTEN's role as a prognostic and predictive biomarker in these cancers. Thus, we have developed and validated a fully standardized, highly sensitive, robust anti-peptide immuno-multiple reaction monitoring (iMRM) assay for precise PTEN quantification.
Methods
An 11-min micro-flow LC-MRM method for the proteotypic PTEN peptide NNIDDVVR was developed and optimized on an Agilent 6495A triple quadrupole mass spectrometer, validated using CPTAC guidelines. Because direct quantitation of PTEN from cell or tissue lysate was not feasible, we generated an anti-peptide antibody to immuno-enrich NNIDDVVR before LC-MRM. The resulting Immuno-MRM assay was used to quantify PTEN in cell lines, fresh frozen- and formalin-fixed paraffin-embedded (FFPE) cancer tissues including patient-derived xenografts (PDX) treated with the chemotherapeutic paclitaxel.
Results
The average recovery of the anti-NNIDDVVR immuno-enrichment was 90%, the average accuracy of the complete iMRM assay was 87%. Our iMRM assay enabled precise quantitation of PTEN concentrations in cell lines, fresh frozen- and FFPE tissues, down to 0.1 fmol/10 µg (of extracted protein), with high inter-and intra-day precision (CV 6.3%). iMRM PTEN concentrations in BC-derived PDXs were consistent (i) across biological replicates, e.g. 0.7±0.0 fmol/10 µg (PTEN-IHC-negative) and 5.7±0.1 fmol/10 µg (PTEN-IHC-high); (ii) across technical replicates, average %CV of 24% for three cores/block; (iii) generally showed the same trend as the IHC classification. For triple-negative BC-derived PDXs treated with paclitaxel, all metastatic PDXs showed a very good correlation (r^2=0.86) between PTEN concentration determined with iMRM and the regression in tumor size.
Conclusions
Our PTEN iMRM method provides a much-needed tool for the validation of PTEN as a clinically relevant prognostic and predictive biomarker in BC.

P10.16

Development of a Parallel Reaction Monitoring Assay for the Quantification of Interferon Alpha Subtypes

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Introduction
Interferon alpha (IFN-α) comprises 12 subtypes which are all important antiviral cytokines. Even though all subtypes bind to the same receptor, they cause different biological effects. Virus infection induces different expression patterns of IFN-α subtypes, resulting in the expression of variable restriction factors, causing inter-individual variation in the immunological outcome. To investigate these variations, as well as the therapeutically relevant effects of the IFN-α subtypes, it is at first important to accurately quantify the individual IFN-α subtypes. So far, the high sequence similarity of IFN-α subtypes hindered the development of specific immunoassays, therefore the quantification of the individual subtypes. Only PCR-based assays have been used to measure the mRNA of the IFN-α subtypes.

Methods
We approached the development of a parallel reaction monitoring (PRM) assay which can distinguish and accurately quantify the 12 individual IFN-α subtypes. For the assay, unique peptides were selected for each subtype and stable isotope-labelled synthetic peptides (SIS) were synthesized. The peptide characteristics and performances in the PRM assay were empirically determined using SIS peptides, recombinant proteins and different background matrices.

Results
Calibration curves were generated for all peptides and the lower limits of quantification were determined in complex matrices. Using this assay, we were able to quantify spiked-in recombinant protein concentrations of interferon alpha subtypes in THP-1 cell conditioned culture medium. We aim to determine the concentrations of the different IFN-α subtypes and therefore the relation between the subtypes and the different antiviral outcome.

Conclusion
Taken together, the assay presented here may have the potential to be used in precision medicine to measure the different subtypes and generate the IFN-α profiles of individual patients. This will allow new insights into antiviral host reactions and could then support the customized medication of patients after virus infection.

P10.17

Cancer SHooting ARrow Proteomics (cSHARP) to Target OnCo-proteogenomic Panels in a Quadrupolar Environment

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Introduction: MS-based proteomics has enhanced our understanding of the disease mechanisms by identifying candidate biomarkers for patient stratification and has a great potential to deliver clinical utility for precision medicine. Nevertheless, analytical challenges remain, including detection of mutation sequence, minute amount of clinical specimens and assay robustness, to achieve the translation process. Collaborative environments among academia and industrial partners as well as multidisciplinary teams across four countries have been established to drive the proteogenomic biomarker transformation into molecular diagnostics, named cSHARP, representing Cancer SHooting ARrow Proteomics.
Methods: A novel intelligent data acquisition mode called “Hybrid-DIA” MS strategy was employed for quantifying onco-proteogenomic molecules such as EGFR and KRAS with mutations.
Results: Our first case study was conducted on NSCLC cell lines such as NCIH1975, PC9 and A549 cells. We first developed a sample preparation strategy integrating membrane protein extraction and multiple protease digestion to generate target peptides covering mutation sites with good MS detectability. Furthermore, a novel chemical conversion of amino acids was introduced to extend the coverage of targeted peptides with extremely hydrophilic and hydrophobic characteristics and minimize the type of protease used. To evaluate the sensitivity of the hybrid-DIA approach, we monitored different EGFR and KRAS peptide variants, including several cancer driver mutations in different samples. The hybrid-DIA workflow enabled detection of EGFR-L858R in NCIH1975 or KRAS-G12S in A549 cells from only 50 ng peptide material, together with quantitative information on thousands of proteins per run. The quantification results revealed heterogeneous expressions of mutant and wild-type EGFR proteins in different cell lines.
Conclusions: This initiative was launched to take the challenge for realizing proteomics assays to complement the gene-centric clinical assays. The pilot study revealed the underestimated real-world challenge for proteogenomic typing of low abundant oncoprotein.

P10.18

Biomarker Monitoring in Body Fluid by High Sensitivity and High Throughput FAIMS-Surequant™ Is Targeted Quantitation

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Introduction: Accurate and reproducible biomarker analysis in tissues and body fluids is paramount to reliable clinical diagnostics, assessment of treatment regimens and pharmaceutical research. The rapid improvement of targeted quantitative proteomic assays over the last decade has caused such assays to emerge as serious candidates in biomarker quantification studies and clinical proteomics[1]. However, established targeted methods still suffer from inadequate sensitivity and insufficient reproducibility in complex samples[2]. In this study, we report the quantification of a panel of biomarkers in would fluid exudates, using a novel internal standard triggered targeted method that exhibits markedly enhanced sensitivity and robustness[3].

Methods: 365 wound dressing samples derived from a cohort of 43 chronic wound and 8 acute healing human patients were probed for the abundance of 9 wound healing biomarkers characteristic for various stages of wound healing. After protein extraction, the wound fluid exudates were analyzed using the recently developed SureQuant™ targeted method on an Orbitrap Exploris 480 mass spectrometer equipped with a FAIMS ion filtering device and coupled to an EvoSep One liquid chromatography system.

Results: We demonstrate the development of a high-throughput assay of 100 samples per day that achieves quantitation of a panel of biomarkers including elusive proteins in complex proteomic studies, such as cytokines TNFα and IL1β. The addition of FAIMS considerably increases sensitivity and data completeness and enables quantification of proteins spanning 6 orders of magnitude in concentration in wound exudates.

Conclusions: Our optimized targeted proteomics assay represents an important addition to state-of-art targeted methods for high complexity proteomic samples and a crucial step towards the ushering of clinical proteomics to the forefront of diagnostics and precision medicine.

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P10.19

Optimization of the protocol for collection and proteomic analysis of exhaled breath condensate for the lung cancer diagnostics

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Introduction: Lung cancer (LC) is a leading cause of cancer mortality. Exhaled breath condensate (EBC) is a promising subject for early LC screening with noninvasive collection Protein biomarkers search is of particular importance and requires the development of standardized protocols of EBC collection and sample preparation for widespread clinical application. A particular sampling modification is proposed in order to overcome the lack of standardized protocols for EBC.
Methods: EBC samples from 20 LC patients were collected with RTube® device (Respiratory Research, Inc., Charlottesville, VA). EBC of healthy controls were used for improvement of biomarkers selectivity. The collecting tube was additionally rinsed with methanol to increase the protein yield. Tryptic peptides were analyzed by HPLC-ESI-MS/MS using a nano-HPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TiMS TOF mass-spectrometry system (Bruker Daltonics, Bremen, Germany).
Results: The methanol rinsing significantly increased the average number of the detected proteins in individual LC samples from 56±29 (±SD) to 85±25. Totally 115 proteins were identified, 32 of them were more often identified in EBC without additional methanol rinsing, while 70 proteins were more often found in the rinses. In addition, the rinsing increased in the detectability of known LC protein markers S100-A9 (from 17 to 18) and dermcidin-(from 16 to 20). Thus, the modernized approach made it possible to identify the oncomarker(s) in individual samples of EBC of LC patients.
Conclusions: The obtained results demostrate a significant sorption of EBC proteins during the collection, which lead to incomplete protein identification. The proposed approach for protein desorption can be effective for different types of collection devices and can be useful for developing standardized protocols for collecting EBC for further proteomic analysis. The research was supported by RFBR grant #18-29-09158MK.

P10.20

A Multi-Faceted System for Differential Glycoprotein Analysis: Toward the "Design Drawings" of GlyCo-targets for the Highly Specific Antibody Drug Development

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Introduction: Membrane glycoproteins are known to alter their glycosylation sites and glycan structures along with changing cell status and environments related to the disease, which have been investigated as targets for the development of more effective antibody drugs with fewer side-effect. For this purpose, the discovery research should be completed by verifying a membrane glycoprotein selected as a candidate with in-depth structural information. This focused analysis allows to produce the immunogen for developing a unique antibody recognizing both glycan and peptide parts of a glycopeptide fragment on the protein. Here, we show a new multi-faceted glycosylation analysis system for validating drug target molecules.
Methods: Two technologies were combined for the glyco-target analysis: 1) a lectin microarray-based system to explore disease-relevant glycosylation alteration in a glyco-target using lesion and surrounding cells isolated from the frozen tissues (0.2 mm^3), and 2) an MS-based in-depth analysis system to explore disease-relevant structures/sites of glycans and their micro-/macro-heterogeneity. More than 600 tissue specimens of 12 diseases were provided and effectively used for technological verification.
Results: We found disease-specific glycan alteration on 32 candidates listed in the discovery phase. We obtained each piece of evidence on 25 candidates by in-depth analysis, whose data was provided as the "design drawings" for making the immunogens. Pathological and biochemical validation is ongoing for 9 of these antibodies to be a "dual-recognizing" antibody against a specific glycopeptide region on a glyco-target.
Conclusions: A multi-faceted glyco-approach of MS with lectin microarray facilitates the validation of the disease-relevant glyco-alteration in a specific membrane-tethered protein using pathological tissue sections, which is expected to contribute to unique antibody-drug development by expanding the repertoire of drug targets. This study was supported by projects for utilizing glycans in the development of innovative drug discovery technologies from the Japan Agency for Medical Research and Development (AMED).

P10.21

Kinome Analysis of CIC-Rearranged Sarcoma Using Peptide Microarray; Global Investigation of Kinases Affected by Culture Condition

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Introduction: CIC-rearranged sarcoma is a highly aggressive mesenchymal malignancies, characterized by the unique fusion genes. The standard therapy has not been established, and the prognosis of the patients with this sarcoma remains dismal. The patient-derived cancer cell lines are pivotal tools for cancer research, and we successfully established cell lines from surgically resected tissues in our previous studies. Using these cell lines, we have conducted drug screening to identify the effective anti-cancer agents for CIC-rearranged sarcoma. The characters of tissue cultured cells are affected by culture conditions, and the monolayer culture is different from the spheroid one in terms of drug response. To explore the differential response to the treatments with anti-cancer drug and to identify the proteomic backgrounds of different response to them, we performed drug screening and proteomic study.

Methods: We used cell lines derived from tumor tissues of the patients with CIC-rearranged sarcomas, and treated them using 214 anti-cancer drugs, whose use was approved in the treatments for different types malignancies. We examined the kinase activities in the cell lines used in this study, using the membrane type peptide microarray, such as PamStation (PamGen).

Results: We detected the anti-cancer drugs, which showed the remarkable anti-proliferative effects on the CIC-rearranged sarcomas. The proteomic study conducted by PamStation revealed the similarity of cell lines without regard the culture types, such as the monolayer and the spheroid ones.

Conclusions: We found several anti-cancer drugs which are worth further investigating toward novel therapy in CIC-rearranged sarcomas. Similarity of kinome profiles between the monolayer and spheroid cultures may suggest the urgent needs to establish the novel culture methods, which will allow the effective pre-clinical study.

P10.22

Evaluation of Humoral Immune Dysfunction in Chronic Lymphocytic Leukemia by Affinity Proteomics.

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Introduction
Chronic Lymphocytic Leukemia (CLL) is a disease characterised by high number of B cells type CLL in blood (≥5x109/L). Genomics and epigenomic studies have shown the significance of: i.-Genomic Alterations: del13q, del11q, del17p, trisomy 12... ii.-Mutational landscape: from IGHV genes to TP53, NOCHT1, ATM... iii.-Epigenomic landscape: Hypomethylation changes in transcription factors which modulate genes involved in BCR signaling, NF-κB activation... and which are present in prior stages (Monoclonal B-cell lymphocytosis -MBL-). Currently, the microenvironment study and the knowledge about immune system, adaptative/innate response and B-cell differentiation have started to become important in the context of the study of CLL.

Methods
Immunomonitoring analysis of soluble proteins involved in the regulation of T and NK lymphocytes was carried out. Also, analysis of growth factors, cytokines and chemokines in relation to the tumor microenvironment and immunoglobulin isotype characterization was performed using Luminex xMAP technology on 11 MBL samples and 56 LLC samples. As a standard sample was used a human plasma pool from National Institute of Standards and Technology.

Using the Standard sample as a threshold, proteins were analyzed according to levels were high/low. Only proteins with up/down concentrations to standard sample in more than 80% of the MBL and CLL samples were considered for analysis.

Results
In this study, 11 plasma soluble proteins present differences in concentrate when MBL and LLC samples are compared. These can be grouped into 4 signaling pathways: Interleukin-10 signaling (IL-10, MIP-3α and MIP-1β), Signaling by Interleukins (IL-7, IL-16 and IL-20), Signaling by GPCR (CXCL1, CXCL13, CXCL5 and SDF-1α) and Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell (Singlec-7). However, no significant differences were found when characterising immunoglobulins or other proteins, in terms of their concentration.

Conclusions
This study suggests that the cellular microenvironment plays a key role in disease progression

P10.23

A Large-Scale Assay Library for Targeted Protein Quantification in Renal Cell Carcinoma Tissues

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Introduction
Renal cell carcinoma (RCC) represents 2.2 % of cancer incidences, however, prognostic or predictive protein biomarkers specific for RCC are generally not available. To support proteomics research of localized and metastatic RCC, we introduce a new library of targeted mass spectrometry assays for accurate protein quantification in malignant and normal kidney tissue.

Methods
Aliquots of 86 initially localized RCC, 75 metastatic RCC and 17 adjacent non-cancerous fresh frozen tissue lysates were trypsin digested, pooled and fractionated using hydrophilic chromatography and analyzed using LC-MS/MS on QExactive HF-X mass spectrometer in data-dependent acquisition mode. The library was generated in Spectronaut software. Two published datasets A-B [1] [2] and two new pilot datasets C-D of localized and metastatic RCC tissues measured in data-independent acquisition (DIA) mode were processed using the new library in Spectronaut.

Results
The newly established assay library contains 77,817 peptides representing 7960 protein groups (FDR=1%). Its application resulted in increased numbers of quantified proteins in datasets A (2463 proteins, +4%) and B (4492 protein groups, >2 fold), with a clear separation of tumor and non-tumor tissues in both studies. Analysis of datasets C of metastatic RCC responding vs. non-responding to sunitinib treatment and dataset D of initially localized vs. metastatic RCC tissues led to consistent quantification of 5181 and 5253 protein groups (FDR=1%).

Conclusions
Application of our spectral library leads to quantification of substantially increased part of RCC proteome. The new library has potential to contribute to better understanding the RCC development at molecular level, leading to new diagnostic and therapeutic targets.

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P10.24

Discovery and Validation of Circulating Autoantibodies Associated with the ACPA Status in Early Rheumatoid Arthritis

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Introduction: The presence of anti-citrullinated protein antibodies (ACPAs) is a highly specific hallmark of rheumatoid arthritis (RA), which is detected also in early disease. However, 20% of RA subjects test negative for ACPA and thus their early diagnosis and treatment may be delayed. In this work, we aimed to use a proteomic strategy to search for plasma autoantibodies associated with the ACPA status that could be useful to assist the early and precise diagnosis of RA.
Methods: The plasma IgG and IgA repertoire of 80 ACPA positive and 80 ACPA negative subjects entering the Epidemiological Investigation of RA (EIRA) cohort was profiled using an antigen suspension bead array built with 260 protein fragments within Human Protein Atlas selected from an initial untargeted screening on planar arrays. A validation phase using a suspension bead array including 27 antigens was carried out on additional EIRA samples including 386 ACPA+, 358 ACPA- and 372 control subjects. Wilcoxon rank sum and Fisher's tests were applied to compare autoantibody levels and reactivity frequencies between groups.
Results: The prevalence of IgG autoantibodies towards Testis-specific Y-encoded-like protein 4 was significantly higher in ACPA- subjects compared to ACPA+ (8% vs. 3%) and controls. Significantly higher IgG autoantibody levels towards dual specificity mitogen-activated protein kinase kinase 6 were also observed in ACPA- subjects, although without significant differences in prevalence. In contrast, we found significantly higher IgG autoantibody levels in ACPA+ individuals compared to ACPA- and controls towards anosmin-1 and muscle related coiled-coil protein. No significant differences were validated at IgA reactivity levels for any of the antigens.
Conclusions: Although further validation in other early RA sample cohorts is needed, our data suggest the measurement of these four autoantibodies might be useful to assist the early diagnosis of RA and give additional insight into its pathogenesis.

P10.25

Integrated Proteomic and Glycoproteomic Signatures of Protein N-Glycosylation Aberrations in Ulcerative Colitis

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Introduction: Patients with extensive ulcerative colitis (UC) have an increased risk of developing colorectal cancer (CRC). Although CRC-associated alterations in glycoprotein have been widely reported, changes in the glycoproteome that are involved in UC to CRC transformation have not been studied thoroughly.

Methods: We performed an integrated proteomic and N-glycoproteomic study to interrogate the progression of UC to CRC. Clinical tissue biopsies from UC patients, including non-dysplastic (NEG), low-grade dysplasia (LGD), and high-grade dysplasia (HGD)/CRC specimens, were investigated to uncover the role of protein glycosylation in the pathological changes of UC progression. Weighted correlation network analysis was applied to construct proteomic and N-glycoproteomic co-regulation networks according to our omics data.

Results: Our results revealed some unique features involving an altered N-glycoproteome associated with the pathological changes from UC to CRC. A strong relationship between N-glycoforms and disease progression was observed. In comparison to NEG and LGD, the relative abundance of fucose and fucose/sialic acid-containing N-glycan was elevated in HGD and cancer tissues. Further, shown in quantitative intact N-glycopeptide analyses, a roster of fucose-containing glycopeptides significantly increased in the HGD/CRC group. Finally, quantitative proteomics analyses revealed upregulation of sialyltransferases (ST6Gal1 and ST6GalNAc1) and galactosyltransferases (GCNT1) as the disease status progressed from NEG to LGD.

Conclusions: Our results provided novel data and perspectives into the potential roles of glycosylation in the pathological processes implicated in UC neoplastic progression. Moreover, the detection of intact glycopeptides, in which glycans conjugated on N-glycosites, could be meaningful and significant for glyco-biomarker development in the context of early detection and intervention of UC associated CRC.

P10.27

Global Immunopeptidomics by Differential Ion Mobility Mass Spectrometry for Identification of Patient Specific HLA-Presented Antigens Directly from Clinical Tissues

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Introduction: Human leukocyte antigen (HLA) binding peptide (HLAp, also known as immunopeptide) with somatic mutation, called as neoantigen, is an optimal target for cancer immunotherapy. However, the global analysis of immunopeptides including neoantigens is still difficult due to a technical limitation i.e., for in-depth analysis, generally a large amount of sample is required. To overcome this hindrance, we employed the high-field asymmetric ion mobility spectrometry (FAIMS) ion source and established the global immunopeptidomic analysis.
Methods: First, a colon cancer cell line HCT116 was used to optimize the method. FAIMS-Pro interface was installed onto Orbitrap Fusion Lumos for the seamless, simultaneous and multiple gas-phase fractionations. Then with whole exome sequencing (WES)-based tailored-database, the method for global immunopeptidomics by differential ion mobility mass spectrometry (DIM-MS) was established. Next, around 40 mg of clinical tissues of stage-IV colon and rectal cancer (CRC) as well as its adjacent normal tissues (n= 17) were analyzed to interrogate immunopeptidome and the patient specific neoantigens at tissue-level.
Results: By DIM-MS, on average, more than 7 neoantigens out of nearly 7,000 HLAps were identified from 1E8 cells from HCT116. Three independent analyses resulted in total 9,249 HLAp including 11 neoantigen identification. From clinical tissues, on average, 5,725 and the total 44,785 HLAps including 2 neoantigens from tumor-origin were identified. Noteworthy, one of which carried cancer driver gene KRAS-G12V mutation. Further, when we compared pairwise immunopeptidome acquired from colon tumor and adjacent normal tissues, tumor-exclusive HLAp exhibited the characteristic distinctions of C-terminus cleavage by tryptophan that indicated the possible association of chronic inflammation in lesions.
Conclusions: Newly established DIM-MS based immunopeptidomic analysis make direct interrogation possible from biopsy-sized tissues and the obtained knowledge will settle the controversial condition of neoantigen-depletion and further reveal the crucial insights for the future innovation of cancer immunotherapy.

P10.29

Immunoproteomics Characterization of Ligustrum Lucidum Pollen Allergens Causing Respiratory Allergies in Polysensitized Patients

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Introduction: Pollen of Ligustrum lucidum (privet hedge) is a relevant cause of allergic respiratory disease worldwide (1). L. lucidum belongs to the Oleaceae family, one of the most allergenic families, including Fraxinus and Olea genus. However, it has been underestimated as a sensitization factor. Previously, we described six novel immunoreactive pollen proteins from L. lucidum responsible for causing a respiratory allergy in mono-sensitive patients (2). Here we explored which proteins causing respiratory allergies in polysensitized patients and are responsible for cross-reactivity between Ligustrum and other Oleaceae genus.
Methods: Total proteins were extracted from Ligustrum lucidum pollen using a modified phenol extraction method (3). Then, proteins were separated by two-dimensional gel electrophoresis (2-DE) for immunoblotting. Sera of 27 polysensitive patients for Ligustrum and Fraxinus pollen was used as a source of the Ig-E antibodies for western blot differential analysis. Immunoreactive protein spots were analyzed by mass spectrometry.
Results: Electrophoretic pattern obtained from L. lucidum pollen revealed proteins in ranges of 17-100 kDa, enriched in a range of 25-75 kDa. The 2-DE profile resulted in approximately 200 spots, and 2-DE immunoblots using a pool of sera from polysensitive patients revealed 27 allergenic proteins. Interestingly, individual immunoblots showed high heterogeneity between polysensitized patients to immunoreactive spots. Allergens identified by mass spectrometry analysis included the following proteins: profilin, enolase, pollen-specific polygalacturonase, glucanase, alanine aminotransferase, triosephosphate isomerase, ATP synthase, and others.
Conclusions: This study has identified novel allergens from L. lucidum pollen essential for accurate allergy diagnosis. The IgE-mediated response in polysensitized patients exhibits a high heterogeneity degree, hence the necessity of proteomics-driven precision medicine.

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P10.30

Autoantigenomics in Neurology: Holistic Characterization of Autoantigen Repertoires Identifies Patient Subgroups and a Novel Target of Autoantibodies in CIDP

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Introduction: Autoimmune diseases are mostly characterized by autoantibodies in the patients' serum or cerebrospinal fluid, representing diagnostic or prognostic biomarkers. For decades, research has focused on single autoantigens or panels of single autoantigens. Here, we broadened the focus by addressing the entire repertoire of antibody-targeted proteins (the "autoantigenome") in a systemic "omics-like" way. As a proof of concept, we use sera from patients with chronic inflammatory demyelinating polyneuropathy (CIDP), a disease of the peripheral nervous system considered an autoimmune disease.

Methods: We screened 43 human serum samples, of which 22 were from patients with CIDP, 12 from patients with other neuropathies, and 9 from healthy controls via HuProt Human Proteome microarrays testing about 16,000 distinct human bait proteins. Autoantigen repertoires were analyzed via bioinformatical autoantigenomic approaches: principal component analysis, analysis of the repertoire sizes in disease groups and clinical subgroups, and overrepresentation analyses using Gene Ontology and PantherDB.

Results: The autoantigen repertoires enabled the identification of a subgroup of 10/22 patients with CIDP with a younger age at onset and a higher frequency of mixed motor and sensory CIDP. Intravenous immunoglobulin therapy responders targeted 3 times more autoantigens than nonresponders. No CIDP-specific autoantibody is present in all patients; however, anchoring junction components were significantly targeted by 86.4% of patients with CIDP.

Conclusions: The repertoire of targeted autoantigens of patients with CIDP differs in a systematic degree from those of controls. Systematic autoantigenomic approaches can help to understand the disease and to discover novel bioinformatical tools and novel autoantigen panels to improve diagnosis, treatment, prognosis, or patient stratification.

P10.31

The Urine Proteome/Degradome Using N-Terminomics with TMPP-Labelling on the Proteome and Peptidome Fractions

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Introduction

The urinary proteome is largely degraded by proteases and common proteomics methodologies are not adapted to precisely characterize the extremities of cleaved proteins. Therefore, N-terminomics by the doublet N-terminal oriented proteomics (dN-TOP) is a suitable approach to fully characterize the urinary degradome. In this way, and in order to highlight potential biomarkers allowing the monitoring of the renal graft stability, the N-terminal labelling of urine proteins and peptides has been optimized.

Methods

To perform our N-terminomics study using N-terminal amines labelling with light and heavy trimethoxyphenylphosphonium (TMPP), (i) urine proteins were cleaned and labelled before being fractionated, washed and analyzed. The challenging part lies in the peptidome analysis that requires the clean isolation of the peptides before labelling and removing of TMPP excess. The (ii) urine peptides fractions was isolated with molecules and salts contained in urine and that can interfere with TMPP labelling and LC-MS/MS analysis. Thus, a protocol using a ProteoSpin Kit was optimized. Our study was conducted on 10 couples of donor/recipient of renal allografts. All samples were analysed on a Qexactive HF-X.

Results

The analysis of the urine proteins fractions allowed identifying from 579 to 1164 unique proteins, while the dN-TOP strategy allowed identifying 1328 N-terminal peptides. In the peptides fractions, our protocol allowed identifying 271 unique N-terminal peptides resulting from the protease activity in urine. Both proteins and peptides fractions allowed highlighting 26 annotation errors of signal peptides and draw conclusions on overrepresented protein sets.

Conclusion

Our study shows the importance of optimizing sample preparation and paves the way of urine N-terminomics with protocols dedicated to the isolation and the labelling of both urine proteins and peptides. It also shows the efficiency of N-terminomics to generate experimental evidences of annotation errors on signal peptides for instance and to provide valuable information of clinical potential.

P10.32

A Multi-Faceted System for Differential Glycoprotein Analysis: Toward the Discovery of Disease-Related Glycosylation Alterations Using Tissue Crude Samples

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Introduction: For the discovery of glyco-targets for medical use, the simultaneous identification of the disease-related glycans and its carrier proteins from crude glycoprotein samples is of crucial importance. For this purpose, a large-scale analysis method for site-specific glycoforms of intact glycopeptides is strongly needed. To facilitate the mass spectrometry (MS)-based analysis, we developed a lectin microarray (LMA)-based glycomics platform to obtain N- and O-glycomic profiles. For MS-based glycoproteomics, we developed a fragmentation-independent glycoproteomics method called "Glycan heterogeneity-based Relational IDentification of Glycopeptide signals on Elution profile" (Glyco-RIDGE). This presentation will introduce the latest progress of the multi-faced system for tissue samples.

Methods: For spatial glycomics, we optimized a laser microdissection-assisted LMA procedure for the analysis of formalin-fixed paraffin-embedded (FFPE) tissue sections. For Glyco-RIDGE analysis for site-specific glycoforms of glycopeptides in tissue samples, the procedures of sample preparation and data acquisition conditions were optimized.

Results: LMA-based analyses provided 451 glycomic profiles with spatial information from 14 FFPE tissues of normal and diseased mice, which were visualized by an online tool called LM-GlycomeAtlas (https://glycosmos.org/lm_glycomeatlas/index). This visualization facilitated differential analysis of LMA data, revealing tissue- and region-specific protein glycosylation in normal mice, as well as cardiac disease-related glycosylation alterations in failing hearts. In large-scale analysis using the Glyco-RIDGE method, over 10,000 site-specific glycomes were identified from mouse six tissues. The multi-faceted system was verified by applying to clinical specimens, resulting in that membrane glycoproteins as glyco-target candidates were successfully identified for 12 diseases including cancer.

Conclusions: These results indicate the feasibility and usefulness of the multi-faceted system for the discovery of glycoproteins with disease-related glycosylation alterations. This study was supported by projects for utilizing glycans in the development of innovative drug discovery technologies from the Japan Agency for Medical Research and Development (AMED).

P10.33

Personalised Phosphoproteomics Identifies Functional Signalling

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INTRODUCTION
Protein post-translational modifications provide a level of cellular control invisible to genetic sequencing, integrating information from the cell and environment rapidly, efficiently and dynamically. Identifying functional phosphorylation amongst the thousands of phosphosites regulated by a perturbation is a major challenge.

METHODS
Here, we introduce "personalised phosphoproteomics", a combination of experimental and computational analyses to link signalling with biological function by exploiting human phenotypic variance. Our approach extracts biologically relevant phosphorylation events by associating the variance in measured phenotypes between humans as they respond to stimuli with dynamic phosphorylation profiles across those same individuals. Our approach does not rely on any a priori phosphosite knowledge, such as existing reported substrates of kinases, making the approach well suited to uncovering biologically relevant links in signalling networks. To employ this method, we generated a comprehensive phosphoproteomics dataset of >11,000 phosphosites in human skeletal muscle biopsies responding to exercise and insulin, to delineate the interaction between these interventions.

RESULTS
We found that phosphoproteomes from different human subjects possesses unique signatures, independent of the proteome. The subject-specific nature of the phosphoproteome was evident even when perturbed by stimuli. We defined variable and invariable phosphosites across subjects. Variable phosphosites included potential biomarkers of phenotypic outliers, confirming previous clinical reports. We applied our personalised phosphoproteomics approach by associating phenotypic variance across subjects with individual dynamic phosphoproteomes. This method identified >100 uncharacterised functionally-linked phosphosites. Many of these phosphosites occurred on proteins intimately involved in our phenotype of interest. This included unexpected communication between mTOR and AMPK, for which we found a role in metabolic regulation.

CONCLUSIONS

These results establish personalised phosphoproteomics as a general approach to investigate the signal transduction underlying complex biology. This brings us closer to understanding the molecular basis of how health outcomes vary dynamically across the human population.

P10.34

Mass Spectrometry-Based Proteomics of Multiple Sites Reveals Signature of Lymph Node Metastasis for Head and Neck Cancer

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Introduction: The presence of lymph node metastasis in the neck is the major prognostic factor affecting patients with head and neck cancer (HNC). Diverse microenvironments are intrinsically connected, contributing to the regulation of tumoral niches. Hence, the integrated investigation of multiple sites can provide a systemic understanding of the molecular landscape of the neoplasm and, therefore, allow for accurate identification of tumor signature of the metastasis.
Methods: Herein, we evaluated the proteome of 140 samples from multiple sites in a 59- HNC patient cohort to reveal insights into the biology and potential biomarkers of locoregional metastasis. By using a quantitative mass spectrometry-based approach in primary and matched lymph node tissues (malignant and non-malignant cells), saliva, and blood samples, we investigated the HNC landscape in a discovery phase using data-dependent acquisition followed by the verification of selected targets in fluids using selected reaction monitoring, and finally, we applied a machine learning model to reveal prognostic markers.
Results: We identified an average of 2,048 protein groups strongly associated with immune modulation across datasets. About 106 differentially abundant proteins from locoregional metastasis in tissue and fluid samples were also strongly implicated in the immune system. The integrated proteome highlighted 15 candidates as prognostic markers that were verified in liquid biopsies and generated high-performance metastasis-dependent signatures.
Conclusions: In summary, we presented the deepest proteome characterization of multiple sampling sites in HNC, thus providing a promising basis for understanding tumoral biology and identifying metastasis-associated signatures.

P10.35

MASTER INFORM Pro - Proteome Profiling for Personalized Oncology

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Introduction:
The Germany-wide registry studies INFORM (enrolling children with relapsed cancers) and MASTER (enrolling young adults with refractory cancers and patients with rare tumors) stratify patients based on genome, epigenome and transcriptome signatures for individualized molecular treatment recommendations. However, data from the functionally and therapeutically important layer of the proteome was previously not included. We established a clinical proteomic workflow enabling the integration of proteomic data into the molecular tumor board (MTB) workflow and report on the quantitative (phospho-)proteome expression profiles of sarcoma patients included in INFORM/MASTER.

Methods:
We implemented a workflow optimized for throughput, reproducibility, limited sample availability and short turn-around time. This was deployed to measuring patient proteomes. Briefly, fresh frozen tissue slices were lysed in SDS buffer followed by SP3 digestion, TMT labelling for multiplexing, basic reversed-phase offline HPLC fractionation and Fe^{3+}-IMAC for deep (phospho-)proteome coverage. Samples were measured by DDA on a Thermo Fusion Lumos and a fully automated pipeline to control for data quality and generate proteome reports for the molecular tumor boards was used for data processing.

Results:
The clinical proteomic workflow enables profiling the expression levels of >7500 protein groups and >20,000 phosphopeptides per patient. We showed that it is feasible to generate and integrate proteome profile reports in time for the MTB meeting. So far, >200 sarcoma patients have been analysed and the data shows that proteomic profiling is indeed feasible in the context of large-scale personalized patient stratification programs. We also have initial indications that adding a proteomic component can add critical information not available from genomics data.

Conclusion:
We show that integrating prospective profiling of cancer patients for full and phosphoproteomes in the framework of the MASTER and INFORM registries of the DKTK is feasible and useful to advance personalized treatment recommendations.

P10.37

Proteomics-Informed Two Stage Model of Resistance in Acute Myeloid Leukemia: Identification of Novel Therapeutic Targets to Inhibit Early Resistance

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Introduction:
The development of new therapeutics for acute myeloid leukemia (AML) has seen numerous recent advancements with eight approved drugs over the past five years. This has included approval of FLT3 inhibitors (FLT3i), such as gilteritinib, for patients with activating mutations of FLT3, which is one of the most prevalent genetic features of AML. While use of FLT3i has led to promising clinical results, resistance and relapse still occur for nearly all patients, creating an urgent need for drug combination approaches that mitigate mechanisms of resistance and extend duration of response

Methods.
Whole exome sequencing, RNASeq, metabolomics, and global and targeted proteomics and phosphoproteomics analyses were applied to AML cell line models of early and late resistance in response to the FLT3i gilteritinib and quizartinib, in the presence or absence of the exogenous ligands FGF2 and FLT ligand. Data were integrated with an emphasis on key regulatory proteins and pathways. Initial observations from the cell line models were verified in AML patient samples with clinical outcomes.

Results
Early resistant cells undergo metabolic reprogramming, grow more slowly, and are dependent upon Aurora kinase B (AURKB). Late resistant cells are characterized by expansion of pre-existing NRAS mutant subclones and continued metabolic reprogramming. The two stage model closely mirrors the timing and mutations of AML patients treated with gilteritinib. Pharmacological inhibition of AURKB resensitized both early resistant cell lines and primary leukemia cells from gilteritinib-treated AML patients. Although many of the metabolic responses were evident at both the mRNA and protein level, the dependence on AURKB was only revealed by the proteomic and phosphoproteomic data.

Conclusions:
These findings support a combinatorial strategy to target early resistant AML cells with AURKB inhibitors and gilteritinib before the expansion of pre-existing resistance mutations occurs.

P10.38

Differential Molecular Signatures in Synovial Membrane and Synovial Fluid from Patients with Rheumatoid Arthritis and Psoriatic Arthritis

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Introduction: The differential diagnosis of Rheumatoid Arthritis (RA) and Psoriatic arthritis (PsA) is often difficult due to the similarity of symptoms and the unavailability of reliable clinical biomarkers. Molecular alterations contribute to the pathophysiological processes in the joint. Therefore, we first aimed to evaluate whether differences in the lipid profiles from synovial membrane (SM) and SF could aid the diagnosis of these diseases.
Methods: SM samples of patients affected by RA (n=6), PsA (n=12) and controls (n=10) were compared using MALDI-Mass Spectrometry Imaging (MSI) on a RapifleX. Next, a targeted approach based on multiple reaction monitoring (MRM-MS) was performed to further validate the lipidomic alterations reported by MALDI-MSI between RA and PsA tissues. In this case, 84 lipid species were analyzed in SF (control donors (n=4), RA (n=21) and PsA (n=27)) on a QTRAP 4000. Principal component analysis (PCA) and discriminant analysis (DA) were used for data interpretation.
Results: Lipid profiles of PsA and RA SM were distinguished by MALDI-MSI followed by PCA-DA. Llipid species, including sphingomyelins, phosphatidylcholines and phosphatidylethanolamines, presented the greatest separation power to classify RA and PsA tissues. The abundance of those with discriminatory potential was compared using ANOVA. This analysis found 35 species significantly different among the groups, most of them significantly increased in RA and PsA compared to controls. The spatial distribution of these species was associated with areas with increased vascularity and inflammatory cell infiltrates. On the other hand, RA and PsA patients were also correctly classified based on the SF levels of all quantified lipid species according to PCA and clustering analysis.
Conclusions: Our study shows a distinct lipid profile between RA and PsA synovium and synovial fluid, and reports potential clinically useful lipid markers for the differential diagnosis of these diseases. These markers also provide additional insight to their pathogenesis.

P10.39

Proteomic-Based Precision Medicine for Companion Diagnostics in Autoimmune Diseases

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Introduction: Rheumatoid arthritis (RA) and Systemic Lupus Erythematosus (SLE) are autoimmune diseases that manifest clinically in different organs and joints. Both diseases are characterized by the presence of autoantibodies. The pathogenesis of both RA and SLE are not yet fully understood and there are no proper biomarkers to diagnose either disease or to monitor disease activity during treatment. Proteomics-based precision medicine and companion diagnostics (CDx) approaches aim to improve diagnostics and prognostics of RA and SLE. We present the application of advanced proteomic profiling using high-density protein arrays and advanced mass spectrometry.
Methods: Biofluid samples were obtained from disease cohorts of RA and SLE patients with different treatment outcomes. Proteomic profiling of plasma samples was accomplished by deep proteomics analysis using LC-MS operated in DIA-PASEF and PRM-PASEF mode. Plasma samples were used for protein array-based autoantigen profiling using Sengenics 1631plex Immunome array technology.
Results: Quantitative proteome profiling of RA and SLE patients identified protein groups associated with disease group and treatment outcome. Application of DIA-PASEF and PRM-PASEF enabled deeper biofluid profiling and fewer missing values. Our findings identify novel molecular markers associated with the clinical subtyping of the four patient groups using both array and MS-based analysis. High-density protein array technology allowed subtyping of patients based on significant differentially expressed citrulline-specific autoantigens in plasma, which highlights the association between circulating autoantigens and ACPA status.
Conclusion: CDx based on advanced MS and high-density protein array technology biofluid profiling, shows great promise to improve the prognostics of autoimmune patients. Our study shows that advanced proteomics approaches can facilitate more patient-specific profiling and support the development of clinical companion diagnostics approaches.

P10.40

Aryl Hydrocarbon Receptor-Interacting Protein Regulates Tumorigenic and Metastatic Properties of Colorectal Cancer Cells Driving Liver Metastasis

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Introduction

Liver metastasis is the primary cause of colorectal cancer-associated death. Aryl hydrocarbon receptor-interacting protein (AIP), a putative positive intermediary in aryl hydrocarbon receptor (AHR)-mediated signaling, is overexpressed in highly metastatic human KM12SM colorectal cancer cells and present in many other colorectal cancer (CRC) cells with high metastatic ability.

Methods

We have used meta-analysis, tissue microarrays, TMT-based quantitative proteomics labelling and gain-of-function experiments both in vitro and in vivo to shed light onto the role of AIP in CRC metastasis.

Results

Using meta-analysis and tissue microarrays, we observed a significant association between high expression of AIP with liver metastasis and poor patients' survival. Based on these findings, we studied cellular functions and signaling mechanisms mediated by AIP in cancer cells. AIP overexpression led to an increase in the tumorigenic and metastatic properties of KM12C (non-metastatic) and KM12SM (metastatic to liver) CRC cells. Through quantitative proteomics we found AIP overexpression caused a significant dysregulation of epithelial-to-mesenchymal (EMT) marker. We confirmed via immunofluorescence and western blot that AIP induced Cadherin-17 activation and the overexpression of several transcription factors. The former, induced the signaling activation of AKT, SRC, and JNK kinases to increase adhesion, migration and invasion of CRC cells as demonstrated by PCR and western blot analyses. In vivo experiments showed that subcutaneous or intrasplenic injection of ectopically AIP expressing KM12 cells induced tumor growth and liver metastasis, respectively. It was especially relevant to find that KM12C (non-metastatic) cells ectopically expressing AIP became metastatic to liver.

Conclusions

Collectively, our data reveal new roles for AIP regulating EMT markers, transcription factors and proteins associated with cancer and metastasis to induce tumorigenic and metastatic properties in colon cancer cells driving liver metastasis.

P10.41

Proteomic Analysis Identifies Unique Signatures in Small Cell Lung Cancer Subtypes.

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Introduction: Small cell lung cancer (SCLC) is an aggressive malignancy representing ca. 15% of all lung cancers. Recent studies suggested distinct molecular subtypes (denoted as SCLC-A, SCLC-N, SCLC-P and SCLC-Y) based on the comprehensive transcriptomic analysis of cancer cell lines, patient-derived xenografts, and genetically engineered mouse models [1]. These subtypes, however, have not been characterized at the proteome level.
Methods: 26 patient-derived cell lines were grown and characterized in vitro (molecular subtype by qPCR, growth pattern). Both the cell pellet and the cell media were subjected to nanoLC-MS/MS analysis (Ultimate 3000 RSLCnano system coupled to Q Exactive HF-X) using label-free quantification, followed by database search and statistical analysis (Proteome Discoverer 2.4, R, Perseus).
Results: Unsupervised class discovery of the samples based on the cellular proteomic profiles strongly mimicked the molecular subtyping, with only one cell line being misclassified. Differential expression analysis resulted in 367 and 17 subtype-specific proteins in the cellular proteome and the secretome, respectively. SCLC-Y cell lines are the most distinct on protein level, driven by the upregulation of cell adhesion and epithelial-mesenchymal transition pathways, as well as by the unique overexpression of 6 secreted proteins involved in immune response pathways. SCLC-A and -N can be described by the upregulation of oxidative phosphorylation and ribosome biogenesis respectively, and both subtypes exhibited clear neuroendocrine attributes. Members of the cell surface receptor signaling pathway were found uniquely overexpressed in SCLC-P cell lines.
Conclusions: This study supports the previously proposed molecular classification of SCLC and outlines prominent proteomic differences across the subtypes, ultimately contributing to the development of new therapeutic strategies that may improve clinical outcome of SCLC patients.
References:
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P10.42

Precision Proteomics in Allergy: Pecan Pollen Allergens

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Introduction: Respiratory allergies are increasing worldwide, particularly as many pollen allergens remains to be identified. Pecan (Carya illinoinensis) is an important cause of food allergy: the proteins Car i 1, Car i 2 and Car i 4 have been isolated from the pecan fruit as food antigens. To date however, allergens derived from the pecan pollen remain to be shown despite strong evidence of sensitization in patients suffering allergic diseases such as asthma and allergic rhinitis.
Methods: Total proteins were extracted from pecan pollen using a modified phenolic extraction method, and, subsequently, proteins were separated by two-dimensional gel electrophoresis (2DE) for both total protein stain (Coomassie Blue) and immunoblotting. A pool of 8 sera pecan-sensitive patients was used to analyze blotted proteins. Protein spots were analyzed by Mass Spectrometry.
Results: The 2-DE protein profile of pecan pollen was resolved into around 350 protein spots. Interestingly, two-dimensional immunodetection using pool sera from atopic patients revealed 18 IgE binding protein spots. The LC-MS/MS detected 17 proteins that participate in several cellular processes, including carbohydrate metabolism, electron transport chain, lipid oxidation, anaerobic energy metabolism, among others.
Conclusions: This is the first study to identify allergens from pecan pollen and demonstrates that proteomics has the potential to accelerate the discovery of allergens causing disease. These findings may lead to the development of new diagnostic- and therapeutic modalities in allergy disease from the framework of precision medicine.

P10.43

Novel Candidate Drugs for Malignant Peripheral Nerve Sheath Tumor Revealed by Mass Spectrometry and Drug Screening Using Patient-Derived Cell Lines

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Introduction: Malignant peripheral nerve sheath tumor (MPNST) is a highly aggressive tumor. Although the incidence of MPNST is about 6% of all soft tissue sarcomas, the lifetime incidence in patients with neurofibromatosis type 1 is up to 10%. The optimal treatment modality is complete resection. However, MPNST is known for high local recurrence and distant metastasis rate, leading to the poor prognosis. Therefore, the development of novel treatment methods are urgently needed. We tried to identify the new candidate drugs by integrating the proteome analysis on MPNST tumor samples and drug screening test using patient-derived MPNST cell lines.
Methods: We examined 41 MPNST cases treated in National Cancer Center Hospital, Japan, during June 2004 to March 2019. The proteins expressed in MPNST tumor samples were comprehensively analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), and exponentially modified protein abundance index (emPAI) value of each protein was calculated. By statistically processing the obtained emPAI values, we tried to identify proteins that were overexpressed in the poor prognosis group. We also conducted a drug screening test by 214 drugs including FDA-approved anti-cancer drugs using five novel patient-derived MPNST cell lines.
Results: We revealed 5292 proteins expressed in MPNST tumor samples by LC-MS/MS. By using the emPAI values of these proteins, we identified 89 proteins that were characteristically overexpressed in the poor prognosis group. The results of the pathway analysis of the 89 proteins and the mechanism of the drugs that exhibited remarkable anti-proliferation effects in the drug screening test showed the same molecular background.
Conclusion: We successfully identified novel candidate drugs for the treatment of MPNST by integrating the proteome analysis and the drug screening test using original MPNST cell lines. We expect the identified drugs will contribute the treatment of MPNST.

P10.44

Patient-Derived Sarcoma Model; Pivotal Research Resource for Proteomics

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Introduction
Sarcoma is a unique rare mesenchymal malignancy, characterized by the diverse histological appearances, complex clinical and molecular features, and rarity such as low prevalence of less than 1% of all malignancies. Such diversity, complexity and rarity make the sarcoma research considerably challenging. To reveal the complex relationship between genotype and phenotype of sarcomas, and discover the novel innovative medical seeds, we conducted proteogenomics analysis. We found a paucity of adequate cancer models is the bottle neck of sarcoma research, and launched a project to generate patient-derived sarcoma models.

Methods
We established cell lines from fresh tumor tissues surgically resected from patients with histologically various types of sarcomas. The established cell lines were extensively characterized, and the anti-tumor effects of 214 agents on them were examined in a high throughput way. Proteogenome data were generated by next-generation sequencing (NGS), mass spectrometry, and membrane type peptide array.

Results
We established more than 60 cell lines and 40 PDXs of sarcomas of more than 20 histological subtypes. We confirmed that the established cell lines retained the original genetic aberrations.The screen of anti-cancer drugs identified multiple drugs, which demonstrated the remarkable anti-proliferative effects. 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. In addition, the effects of molecular targeted drugs were not always parallel to the mutations. 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.

P10.45

Urinary Proteins RAD23B and CORO1C Associated with Colorectal Cancer Progression and Metastasis

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Introduction Colorectal cancer (CRC) is characterized by diffuse infiltration of tumour cells into the regional lymph nodes and metastasis to distant organs, and its highly invasive nature contributes to disease recurrence and poor outcomes. However, the molecular mechanisms underlying CRC cell invasion remain incompletely understood.

Methods We adopted a staged discovery-verification-validation pipeline in more than 600 urine and tissue samples from healthy controls and CRC patients with a distinct metastatic risk to comprehensively discover and validate noninvasive biomarkers in urine. The performance of the signatures was evaluated and compared with that of serum CEA. Finally, the expression of key urinary protein was validated in tissue specimens, and the function was investigated in vivo and in vitro.

Results We identified the upregulation of DNA damage repair-related protein RAD23B and showed that it associates with CORO1C to facilitate invasion. The tissue validation and functional study showed that RAD23B and CORO1C were associated with distant metastasis and enhanced the invasion and metastasis of CRC cells via a novel integrin/FAK/SRC and relevant pathways. RAD23B interacted and co-localized with CORO1C, and CORO1C aggregated toward the margin of cancer cells in both CRC cells and tissues when RAD23B overexpressed. Mechanistically, overexpression of RAD23B and/or CORO1C further increased invadopodia formation and matrix degradation in SW480 and HCT8 CRC cells. Conversely, silencing of RAD23B expression suppressed tumorigenesis and liver metastasis in xenotransplant murine models. Furthermore, we identified a strong correlation between higher levels of cytoplasmic expression of RAD23B, and poor prognosis and liver metastasis in CRC patients.

Conclusions Our findings provide novel urinary protein biomarkers and potential interventional targets to reliably detect CRC, especially in patients with metastatic CRC. The novel RAD23B-CORO1C signaling axis in CRC cell invasion and metastasis may be of clinical significance.

P10.46

Development of a Multiplexed Protein Panel Using a Targeted-Proteomics Approach for the Study of Resistance to CDK4/6-Inhibitors in Breast Cancer

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Introduction
Recurrent and metastatic disease limit the survival of patients with breast cancer. Despite the improved disease control with CDK4/6-Inhibitors (CDK4/6I), not all patients respond to such therapy. 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 potentially central to CDK4/6I resistance.
Methods
We developed Multiple Reaction Monitoring (MRM)/MS methods for 25 target proteins from the CDK/RB/E2F-pathway 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 method 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 cancer biopsies. A human breast cancer cell line was used as a model during method development. Proteins from cell lysates were isolated, reduced, alkylated and digested with trypsin. The resulting peptides were fractionated into 60 fractions and pooled into 24 fractions. The nano-LC-MS/MRM-assays were used for peptide detection and quantification.
Results
The micro-flow fractionation method coupled to our a highly specific MS-based multiplexed assay with peptide standards, allowed us to work on limited amounts of samples (60 �g), increasing the possibility of detecting low abundance proteins such as cell cycle components. The high-pH micro-flow fractionation method allows us to obtain an average 7.3-fold signal increase compared to an unfractionated sample. We are able to identify and quantify 20 proteins from our panel: CDK1,CDK2,CDK4,CDK6, Cyclin B1, Cyclin D1,Cyclin D3, Cyclin E1, RB1, E1F-1,E2F-3, E2F-4,E2F-5,ESR1, TOP2A, TYMS,EZH2, MKI67,BIRC5,FAT1.

Conclusions
Through the analysis of a panel of cell lines with different CDK4/6I sensitivities we were able to identify differences in protein expression, and to begin to reveal potential markers of CDK4/6I sensitivity.

P11.01

Proteograph Analysis Suite: A Cloud-Scalable Software Suite for Proteogenomics Data Analysis and Visualization.

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[1]*Seer*

Researchers are increasingly adopting multi-omics approaches to understand the complex biological processes that underlie human diseases. Next generation sequencing (NGS) is widely used for identifying genetic variants and gene function while mass-spectrometry is used to quantify protein abundances, modifications, and interactions. A new plasma profiling platform, the Proteograph Product Suite was developed that leverages multiple nanoparticles with distinct physiochemical properties to provide deep plasma proteomic analysis at scale. Here, we present a cloud-based, data analysis software platform called Proteograph Analysis Suite (PAS) that analyzes proteomics data derived from the Proteograph along with genomic variant results imported from NGS experiments.

The PAS features include an experiment data management system, analysis protocols, an analysis setup wizard, and tools for reviewing and visualizing results. PAS can support both Data Independent Analysis (DIA) and Data Dependent Analysis (DDA) proteomics workflows and is compatible with widely accepted format of variant call files from NGS workflows. Data includes; various quality control metrics like peptide/protein group intensity, protein sequence coverage, relative protein abundance distribution, peptide and protein groups. Various visualizations including principal component analysis, hierarchical clustering, and heatmaps allow intuitive identification of dataset trends. Differential expression tools such as volcano plots, protein interaction maps and protein-set enrichment provides functional insights.

Proteomics and genomics data analysis requires a wide collection of different tools, which requires command-line interfaces and operating system-specific requirements that can act as a barrier for researchers to adapt new data analysis tools. Here, the 141 Proteograph plasma dataset1 was loaded to PAS and database search was performed (tryptic; CID/HCD fragmentation; 25 ppm fragment and precursor tolerance; FDR threshold 0.01). 21,959 peptides and 2,499 protein groups were identified. This search was launched through the user interface requiring only 3 clicks. In the background, this search provisioned 142 servers and completed in approximately five and half hours.

P11.02

HPPInspector: Automated Community-Scale Validation of Novel Protein Discoveries

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Introduction:

Global public data enabled the milestone release of the HUPO Human Proteome Project (HPP) blueprint of the human proteome, but also revealed critical needs for rigorous evaluation of community-scale knowledge derived from proteomics big data from multiple sources and experimental protocols. We propose HPPInspector to (a) automatically assess the community-scale significance of novel protein discoveries, and (b) to prioritize tissues, experiments and datasets by their preliminary detection of proteins still missing from the human proteome.

Methods:

HPPInspector reevaluates the significance of search results by imposing spectral quality requirements (using >1M synthetic peptide spectra) and enforcing community-consensus HPP criteria for uniqueness and quality of peptide identifications. HPPInspector also implements modified protein FDRs integrating HPP criteria directly into the FDR calculations, thereby adjusting FDR separately for proteins that do or do not meet HPP criteria for protein discovery. Interactive and shareable results views then allow for detailed inspection of results from proteins to PSMs.

Results:

We used HPPInspector to check proteins that can be called from a set of 37 cHPP and proteome-scale datasets with a total of over 230M spectra corresponding to a total of 264 total searches yielding over 57M PSMs. While the union of results for these datasets appears to detect 16,997 blueprint proteins (94% of neXtProt PE1) and 712 novel (PE2-4) proteins, rigorous inspection with HPPInspector reveals that only 15,260 blueprint and 28 novel proteins are actually supported by the data. However, HPPInspector also detects evidence for over 1,000 proteins that don't yet fully meet HPP criteria, but whose detection in specific tissues and datasets can inform the design of additional experiments (or reanalyses) seeking to detect additional evidence for these still-missing proteins.

Conclusions:

HPPInspector supports community-scale validation and discovery of novel human proteins.

P11.03

Proteome-Wide Analysis of Turnover Rates with TurnoveR and Skyline

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Introduction: While the measurement of protein turnover is relevant in many biological settings, including neurodegeneration and aging, protein turnover studies remain computationally difficult for most scientists. Here, we introduce TurnoveR, a versatile computational tool that performs the full turnover analysis computational pipeline from a metabolic labeling study on the widely accessible, open-source Skyline proteomics platform.

Methods: To perform in vivo estimation of protein turnover rates in mice, we analyzed two completely independent experiments aimed at determining protein turnover rates in mouse liver and skeletal muscle to study the effects of calorie restriction and sarcopenia, respectively. In both studies, mice were metabolically labeled with deuterated leucine supplemented in the diet and processed for mass spectrometry analysis. Samples were analyzed by data-dependent acquisition (DDA) an Orbitrap Velos mass spectrometer and TripleTOF 6600.

Results: Within Skyline, TurnoveR executes a computational pipeline that deconvolutes overlapping heavy/light isotope envelopes, calculates relative isotope enrichment, performs regressions, statistically compares treatment groups, and visualizes results. We re-analyze data in calorie restricted and ad libitum-fed mice to show this approach recapitulates turnover rates and differential changes in turnover between treatment groups calculated in previous studies using previously established tools. Our pipeline confirmed that calorie restricted mice have 13% less newly synthesized protein globally compared to control mice after 20 days of labeling ($p = 2.09e-20$) and have slower turnover of previously reported key mitochondrial proteins such as Echs1 ($p = 0.01$), Mdh2 ($p < 0.0001$), and Got2 ($p < 0.0001$). The calculated fractions of all proteins of all proteins that were newly synthesized were consistent with previously reported values generated by the Topograph tool ($r = 0.91$).

Conclusions: We anticipate that the addition of this external tool to Skyline will facilitate wider utilization of protein turnover analysis in highly relevant biological models, including aging, neurodegeneration, and skeletal muscle atrophy.

P11.04

DeepLC Can Predict Retention Times for Peptides That Carry As-Yet Unseen Modifications

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Introduction: The inclusion of peptide retention time prediction promises to remove peptide identification ambiguity in complex LC-MS identification workflows. However, due to the way peptides are encoded in current prediction models, accurate retention times cannot be predicted for modified peptides. This is especially problematic for fledgling open modification searches, which will benefit from accurate retention time prediction for modified peptides to reduce identification ambiguity. We here therefore present DeepLC, a novel deep learning peptide retention time predictor utilizing a new peptide encoding based on atomic composition that allows the retention time of (previously unseen) modified peptides to be predicted accurately.
Methods: DeepLC uses a convolutional deep learning architecture that is optimized to generalize for a wide variety of modifications. This architecture is then trained on twenty different data sets and its ability to predict retention times for modifications is evaluated.
Results: We show that DeepLC performs similarly to current state-of-the-art approaches for unmodified peptides (R>0.98), and, more importantly, accurately predicts retention times for modifications not seen during training (e.g., for propionyl mean absolute error improved from 462 to 66 seconds). Moreover, we show that DeepLC's ability to predict retention times for any modification enables potentially incorrect identifications to be flagged in an open modification search of a wide variety of human tissue proteome data.
Conclusions: DeepLC is able to accurately predict retention time of even unseen modified peptide. This accurate model enables integration in open modification search engines to increase the number and reliability of identifications. DeepLC is available under the permissive Apache 2.0 open source license and comes with a user-friendly graphical user interface, as well as a Python package on PyPI, Bioconda, and BioContainers for effortless workflow integration.

P11.05

The Development of New Tools to Facilitate Proteomics Data Analysis; the UniProt Proteins API.

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Introduction
To gain a thorough understanding of the data and proteins of interest, analysis of proteomics data is reliant on high-quality protein sequence databases and the ability to query large scale datasets for reliable protein function and features. UniProt is a comprehensive, expert-led, publicly available database of protein sequence, function and variation information. Collaborations with a variety of sources in addition to expert-led manual curation of published research results in a comprehensive database of protein knowledge. The UniProt Proteins API facilitates access to the whole UniProt database for both programmatic and wet-lab researchers in an easy to use, free to access and completely downloadable format.
Methods
The UniProt Proteins API allows users to probe the UniProt database via a multi-query search form or programmatically, this allows researchers from a broad range of backgrounds 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 walk-through will showcase the functionality and query interface that allows large-scale biological data retrieval without needing in-depth knowledge of programmatic languages. This use case example includes instances of how the UniProt Proteins API can be queried using results from proteomics datasets to return biological functional data, protein sequence, protein-protein interaction, and disease variant data. The new functionality of access to post-translational modification data via the API will also be introduced.
All data are freely accessible from www.uniprot.org
The UniProt Proteins API is available at; http://www.ebi.ac.uk/proteins/api/doc

P11.06

Comprehensive Cancer Tissue-Specific Neural Network Spectral Reference Library (SRL) Generation Using DIA-MS Acquisition

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Introduction: Data Independent Acquisition (DIA) strategies such as SWATH are now an integral part of proteomics studies involving large cohorts of clinical samples. After acquisition, DIA-MS data is traditionally analysed using spectral reference libraries (SRLs) created using Data Dependent Acquisition (DDA). The use of SRLs generated using DIA rather than DDA has not been evaluated. Novel tools have emerged for processing DIA data that involve machine learning, such as DIA-NN, which enables new directions. Our aim was to compare the performance of SRLs derived from either DIA (via DIA-NN) or DDA acquisition modes.

Methods: We used 1,261 fresh frozen cancer samples encompassing 73 cancer types from 27 tissue types. Samples were processed and acquired in three Triple TOF 6600 MS instruments in technical triplicate (one run per MS). Samples were grouped based on histopathology and were combined to produce 39 separate pools. Each pool was fractionated using high-pH RP-HPLC (15 fractions) and data was acquired for each in either DIA or DDA mode (39x15x2 runs). Conventional DDA-acquired SRLs were produced using Protein Pilot/PeakView, while DIA-acquired SRLs were produced using DIA-NN.

Results: On average our new DIA-SRL approach improved the number of proteins by 40%, whilst a high degree of overlap was maintained. We then applied specific DIA-SRLs to a relevant cohort of tumours. PCA showed that DIA-SRL segregated fresh frozen tumours from their surrounding normal tissue. We examined sample fixation methods and compared two further cancer cohorts of 118 FFPE with 172 fresh frozen samples. Again, the DIA-SRL differentiated tumour from normal tissue.

Conclusions: This DIA-SRL approach is a new way to generate SRLs using SWATH, rather than DDA acquisition, that does not require changing the instrument or acquisition mode. It can effectively generate tissue-specific libraries that outperform conventional DDA-SRLs, irrespective of organ of origin or tissue preservation technique.

P11.07

Tissue Type Prediction Reveals Protein Expression Patterns

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Introduction
Tissues have their own specific biological function. To achieve that function, the tissue expresses the right protein at the right time. This results in tissue-specific protein expression patterns allowing for tissue classification. Machine learning models can learn these complex protein patterns. This offers various opportunities from comparing the proteomic composition of healthy versus diseased tissues to tracing tissue leakage proteins back to their tissue of origin.
Methods
A total of 217 PRIDE projects were searched with ionbot [1] and tissue annotation was manually added. The data was annotated on the level of (i) 63 tissues, (ii) 88 cell types and (iii) disease status. Healthy data were randomly split into 85 % and 15 % for the train and test set, respectively which was used to train an XGBoost model on protein abundances to classify samples in tissues and cell types. Subsequently, the feature importance as F-scores is used to analyse the most discriminating protein abundances.
Results
With only protein abundance, the model was able to predict tissues with 94,5% accuracy and cell types with 90,1% accuracy. We identified approximately 2000 proteins crucial for classification, which accounts for 17% of the total amount of proteins present in the data. Additionally, one-vs-all classification provided insight into the most important proteins per tissue.
Conclusions
Public proteomics data and state-of-the-art machine learning algorithms allowed for highly accurate classification models for tissues and cell types. Furthermore, the models allowed for revealing the protein expression patterns of these classes. Future research will include peptide modification data thus allowing even higher accuracy and understanding of protein expression patterns. Moreover, the model will be applied to the non-healthy disease statuses to obtain biologically relevant insights.
References
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P11.08

Leveraging Large-Scale Comparative Proteomics across the Tree of Life to Improve Human Disease Models

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Human diseases are typically thought of in the context of organs or tissues. However, at the root of every human disease lies molecular dysfunction of a biological process or protein complex. Despite the revolution in multi-omics data acquisition, the molecular mechanisms underlying genetic diseases remain only partly known. Proteins interacting in the same biochemical complex are often linked to similar genetic traits. Moreover, previous studies have shown that evolutionarily conserved (ancient) proteins are enriched for disease traits and are abundant across human cell types and tissues. A significant portion of these deeply conserved genes are known to be responsible for a large and diverse subset of major human diseases, spanning developmental disorders (e.g., Leigh syndrome, microcephaly, neural tube defects), cancers (e.g., leukemia, breast cancer, colorectal cancer), chronic respiratory diseases (e.g., ciliary dyskinesia, asthma), neurological disorders (e.g., encephalopathy, schizophrenia, autism) and motor dysfunction (e.g., dystonia, spastic paraplegia).

These human genes are traceable to a last eukaryotic common ancestor (LECA), along with many others that remain poorly characterized. Using a co-fractionation mass spectrometry approach, we measured tens of thousands of protein interactions in 31 eukaryotes and 5 prokaryotes (3 bacterial and 2 archaeal species acting as outgroups). We combine this data with phylostratigraphy and machine learning to reconstruct LECA's likely protein complement and those proteins' likely organization into multiprotein assemblies. This strategy will help us potentially discover disease associations and new functions for poorly characterized human genes, thus helping to better characterize the human proteome.

P11.09

Power of prediction: MS²PIP and DeepLC-based rescoring dramatically boosts immunopeptide identification

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Introduction
Immunopeptidomics aims to identify peptides that are presented on major histocompatibility complexes by the immune system. These identifications can then be used to develop vaccines against pathogens and diseases such as cancer. However, immunopeptidomics data analysis pipelines have some major hurdles to overcome, mostly resulting from the non-tryptic nature of immunopeptides. Previously, the machine learning tools MS²PIP and DeepLC have been shown to improve tryptic peptide identification rates by using accurate fragmentation spectrum and retention time predictions to rescore peptide-spectrum matches (PSM) in Percolator. However, MS²PIP showed a decreased accuracy when predicting non-tryptic peptides, such as most immunopeptides. To enable MS²PIP-based rescoring of immunopeptide PSMs, we have developed a highly accurate MS²PIP model for both tryptic and non-tryptic peptides.
Methods
Publicly available immunopeptide mass spectrometry data sets were used to train and test new MS²PIP models specifically for immunopeptides. Spectra from chymotrypsin-digested peptides were also added to the training data to improve predictions for other non-tryptic peptides. Next, immunopeptide PSMs from various datasets were rescored to evaluate the benefit of accurate spectrum predictions on immunopeptide identifications.
Results
The newly trained models drastically improve both immunopeptide and tryptic peptide spectrum predictions. The chymotrypsin-digested peptides further improved prediction accuracy for other non-tryptic peptides. By rescoring immunopeptide PSMs with the new MS²PIP model, consistently over 40% more spectra and 30% more unique immunopeptides were identified compared to conventional Percolator rescoring. Furthermore, rescoring with peak intensity predictions also allowed identifications at a more stringent false discovery rate (FDR) of 0.001, which would otherwise result in no identifications.
Conclusion
We have demonstrated that leveraging MS²PIP spectrum predictions during immunopeptide PSM rescoring results in vastly improved identification rates and allows more confident FDR thresholds to be set. These methods show great promise to substantially improve the downstream identification of novel neo-epitopes in existing immunopeptidomics workflows.

P11.11

Novel Statistics Tools for Reliable Proteome-Wide Quantification of Post-translational Modifications

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Introduction:
Many proteomics experiments are designed to answer a question rooted in differential protein quantification. One might want to know how a certain drug affects protein expression levels. For this differential quantification, potent statistics tools are needed that can robustly and reliably answer these questions. A myriad of these already exists, like our own msqrob1. However, the feat of differential quantification has been made more difficult by the advent of open modification search engines. Suddenly, there are post translational modifications (ptms) that have to be taken into account. This opens up the possibility to do differential expression analysis on the ptm level, thus gaining more insight into the biology of proteins. This project tries to develop novel statistics tools that can do just that.
Methods:
Our own statistics tool msqrob is being updated to be able to handle the modification heavy data coming from our in house open modification search engine ionbot2. Different statistical models are being researched, these include mixed models, general linear regression models and possible combinations. Once it is ready, the final model will be plugged into msqrob.
Results:
A first adaptation of the msqrob framework has been applied to a dataset regarding histone proteins which has provided promising results. However, further benchmark testing needs to be done in order to draw clear conclusions.
Conclusions:
Much work remains to be done, but the resulting application will be a very useful addition to the field. In order to reach the end users, it will be necessary to include a good documentation, and include the new msqrob version into the ionbot cloud environment, to provide easy access and prevent the statistical terminology from being a deterrent to users.
References:
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P11.12

ADPR Classification using DPA Clustering Algorithm

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Introduction: During the last decade, it became clear that the PTM ADP-ribosylation (ADPR) plays an essential role in many biological processes[1]. However, ADPR detection and the mapping of their acceptor amino acids remains a significant challenge because of the labile nature of the modification and the large number of potential amino acid acceptor. The use of Machine Learning (ML) techniques to accurately identify and classify ADPR modifications could increase detection and the potential discovery of new acceptor sites.

Methods: Data is acquired at the FGCZ using the high-resolution Orbitrap mass spectrometry, and stored at the B-Fabric data management system. The tandem mass spectra are extracted using the rawrr[2] package and used to compute features including those designed to identify APDR peptides. We analyze those features using DPA[3], an unsupervised density-based clustering that allows for the automatic classification of spectra into clusters without requiring dimensionality reduction techniques.

Results: Some features show a high discriminative power in identifying ADPR spectra, also confirming preliminary classifications obtained by mass spectrometry experts' manual inspection. The DPA clustering can capture differences and unexpected variations in the spectrum properties, organizing spectra into clusters. This method further provides robust and visual information about the groups, their statistical reliability, and their hierarchical organization. We validated the results using the ground-truth obtained with multiple database search engines.

Conclusions: We show how ML techniques can support the detection and discovery of ADPR, reducing labor-intensive manual curation of large amounts of spectra.

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P11.13

A Transformer for Prediction of MS2 Spectrum Intensities

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Machine learning has for a long time been an integral part of the interpretation of data from mass spectrometry-based proteomics. Relatively recently a machine-learning structure appeared that has successfully been employed in other areas of bioinformatics, Transformers. One of their key properties is that they enable so-called transfer learning, i.e. adapting networks trained for other tasks to new functionality with relatively few training examples.

Here, we implemented a Transformer based on the pre-trained model TAPE for the task of predicting MS2 intensities. TAPE is a general model trained to predict missing residues from protein sequences. Despite being trained for a different task, we could modify its behavior by adding a prediction head at the end of the TAPE model and train it using the spectrum intensity from the training set to the well-known predictor Prosit.

We demonstrate that the predictor, which we call Prosit-Transformer, is outperforming the recurrent neural network-based predictor Prosit, increasing the median angular similarity on its hold-out set from 0.908 to 0.923.

We believe that transformers will significantly increase prediction accuracy for other types of predictions within mass spectrometry-based proteomics, particularly predictions that use amino acid sequences as input.

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P11.15

Extending INFERYS' Capabilities to CID and TMT Data for (Non-)Tryptic Peptides

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Introduction

Rescoring approaches make the intensity dimension usable for peptide identification by comparing experimental spectra with predicted peptide fragment ion intensities. Recently, we introduced Inferys, a deep learning framework which accurately predicts collision-energy-dependent fragmentation spectra on consumer hardware without the need for GPUs. The 2021 release of Inferys incorporates major architectural changes, resulting in improved inference speed and vastly increased capabilities in a single model.

Methods

The new Inferys architecture is based on Transformers and employs attention layers while retiring the Prosit-derived sequence-to-sequence approach. The training data comprises ~12M high-scoring spectra from all published ProteomeTools peptides. Inferys and the corresponding Rescoring and Spectral Library Generation workflows will be available in Proteome Discoverer 3.0.

Results

Inferys now utilizes custom implementations of advanced neural network layers (e.g. Multi-Headed Self-Attention), which allow a reduction of the model size by ~10-fold, resulting in ~3-fold faster predictions on a CPU. The resulting single model is more accurate on previously supported data types across all sequence lengths, charges and terminal amino acids, as well as for internal basic residues (median spectral angle (SA) of ~0.91 overall). Predictions are substantially improved for non-tryptic peptides (median SA ~0.90) and singly charged peptides (median SA ~0.90). Predicted spectra for TMT-labelled peptides achieve comparable accuracy as predicted spectra of non-labeled peptides (median SA ~0.90), while CID spectra are slightly less accurate (median SA ~0.85).

Utilizing this improved Inferys model for Rescoring workflows cuts the required compute time in half and increased identifications for CID data and HLA peptide data by more than 10% at 1% FDR or achieved more identifications at 0.1% FDR compared to a 1% FDR cutoff without rescoring.

Conclusions

Here, we extend Inferys to support CID and TMT data and substantially improve prediction accuracy for non-tryptic and singly-charged peptides, while boosting inference speed.

P11.16

InfineQ: Real-time Cloud-Based DIA Data Processing For High-Throughput Proteomics

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Introduction
Data Independent Acquisition (DIA) holds an immense promise for biomarker discovery due to increased protein identification, high reproducibility and low number of missing data. However, large number of samples are needed to gain statistical power needed for true biomarker discovery and signal processing of collaborative high-throughput proteomics experiments remains a challenge. InfineQ overcomes this problem by enabling real time, parallel data processing in the cloud with collaborative functionality.

Methods
InfineQ is based on DIA-NN which exploits deep neural network and signal extraction strategies for DIA data. Because of InfineQ's cloud solution, data processing can now be parallelized, leading to much shorter data processing times and removing the limit on the size of the cohort. We use a serverless k8s approach to split each run into multiple pieces which are processed in parallel. On top of this the code is optimized for efficiency and speed of processing. Because the scalability bottleneck is removed, additional algorithms can be added improving quality of outcome without any observable time impact. Multiple groups can work on the same project with secured centralized storage of data, quality control of runs and single pipeline environment improving collaboration and reproducibility of results.

Results
Run time of a single DIA files is brought down to 4 minutes and recently InfineQ processed 10,000 files within 6 hours.
For the users, all internal workings such as calibrations are done automatically without the need for spike-in peptides. Integrated cross-run alignment with FDR control treats data as single cohort, reducing the number of missing values and decreasing the FDR. API allows programmatic access to InfineQ from Jupyter notebook and the possibility to interrogate the results directly on the cloud.

Conclusions – InfineQ is the first cloud-based DIA data processing software with collaborative capabilities and unprecedented speed and scale needed for biomarker discovery

P11.17

MAGPIE: A Machine Learning Approach for Deciphering Protein-Protein Interactions in Human Plasma

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Introduction
Immunoprecipitation coupled to tandem mass spectrometry (IP-MS/MS) methods are often used to identify protein-protein interactions in biological samples. While these approaches are prone to false-positive identifications through contamination and antibody non-specific binding, their results can be filtered by combining the use of negative controls and computational modelling. However, such filtering does not effectively detect false-positive interactions when IP-MS/MS is performed on human plasma samples, given an overwhelming propensity for non-specific interactions. Therein, proteins cannot be overexpressed or inhibited, and existing modelling algorithms are not adapted for execution without such controls.

Methods
Herein, we introduce MAGPIE, a novel machine learning-based approach for identifying interacting proteins in human plasma using IP-MS/MS. MAGPIE leverages negative controls that include antibodies targeting proteins not known to be present in human plasma to detect false-positive interactions. Unsupervised learning algorithms are first applied to label-free mass spectrometry quantification data to identify a set of high-quality negative controls that can be used for false-positive interaction modelling. MAGPIE then uses a logistic regression classifier to assess the reliability of interacting proteins detected in IP-MS/MS experiments using antibodies targeting known plasma proteins.

Results
When applied to five IP-MS/MS experiments, targeting four different plasma proteins, MAGPIE identified 68 protein-protein interactions with an FDR of 20.7%. Our algorithm significantly outperformed a state-of-the-art tool for standard protein-protein interaction discovery, SAINT, detecting 3 times as many interactions at half the FDR. Interacting proteins identified by MAGPIE are further supported by known and predicted interactions in the STRING protein interaction repository. Finally, protein-protein interactions deemed of high-confidence by our tool show a significantly higher level of co-expression as reported by COXPRESdb than unreliable ones, further highlighting the quality of MAGPIE's assessment.

Conclusion
MAGPIE provides an unprecedented ability to detect human plasma protein-protein interactions, enabling a better understanding of biological processes taking place in plasma.

P11.18

PepGM: A Probabilistic Graphical Model for Taxonomic Profiling of Viral Proteomes and Metaproteomic Samples

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Introduction: Taxonomic inference in mass spectrometry-based metaproteomics is a complex task. The presence of proteins and corresponding taxa must be inferred from a list of identified peptides which is often complicated by protein homology: many proteins do not only share peptides within a taxon but also between taxa. Correct taxonomic identification is crucial when identifying different viral strains with high sequence homology – considering, e.g., the different epidemiological characteristics of the various strains of SARS-CoV-2. Our work therefore aims for accurate viral strain identification.
For protein inference, the peptide-protein relationships can be represented as bipartite graphs. Probabilistic graphical models have been used successfully to propagate peptide scores to the protein level. However, similar methods are not yet available at the peptide-taxon level where uncertainty about the species present adds an additional level of complexity. Instead, current approaches rely on strategies such as peptide-spectrum-match counting or the use of unique peptides.
Methods: In our PepGM approach, we represent the peptide-taxon relationships as a bipartite graph where two types of nodes represent peptides and taxa, respectively. The resulting structure serves as scaffold for a factor graph, allowing for the computation of the marginal distributions of peptides and taxa. Propagation of peptide scores to taxa takes place through a message passing algorithm and results in taxonomic identifications with a corresponding statistically sound score. PepGM is implemented in python.
Results: This graphical model is evaluated with viral and metaproteomic mass spectrometric data sets. It shows good taxonomic resolution at species level.
Conclusion: Our PepGM approach will support the statistically sound inference of taxa in mass spectrometric datasets and eliminate the need for error prone heuristics.

P11.19

Deep Plasma Proteomics at Scale: a Machine Learning Enhanced Multi-Nanoparticle Approach to Improve the Depth of Plasma Proteome Coverage

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Blood plasma is the ideal biospecimen to assess the health and diseased states of humans. However, the wide dynamic range of the plasma proteome limits in-depth coverage in large-scale proteomics studies with current technologies. Here we have developed a fast and scalable technology that employs intricate protein-coronas formed on the surface of engineered nanoparticles (NPs) to enhance the depth of plasma proteomes. A panel of 5 engineered NPs allows rapid quantification of thousands proteins across 7 orders of magnitude from plasma with high precision. The key to expand proteomics applications of NP is to characterize physicochemical properties driving protein corona formation while exploring biological pathways interrogated with each NP.

We have engineered and tested a set of functionalized NPs with specific physicochemical properties and profiled plasma proteomes determining differentially enriched proteins with LC-MS/MS analysis. Based on the quantitative differences, we have modeled protein intensities and abundances of protein families as a function of NP's physicochemical makeup.

Proteins are differentially sampled by specific physicochemical characteristics of the NPs including charge, hydrophobicity, and specific chemical groups. This allows NPs to sample the proteome at the proteoform level across a wide dynamic range by affinity and concentration. Our data exemplifies how NPs can be further optimized to interrogate proteins across biological pathways and facilitate unbiased and broad proteome coverage. Our data allows us to design and engineer NPs to capture proteins in plasma broadly or optimize NP panels for specific protein families, PTMs or other molecular classes for next generation large-scale omics studies and biomarker discovery.

P11.20

Evolution of Protein Functional Annotation: Text Mining Study

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Introduction: In March 2018, the HUPO C-HPP consortium launched the neXt-CP50 project, in which CP stands for "characterization of protein" and uPE1 refers to the uncharacterized PE1 proteins in neXtProt. There are currently 1669 PE1-PE4 entries which have no functional information.
Methods: To make our own predictions, we used text-mining approach and compared it with the results of computational prediction of protein functions, obtained using other instruments, eg. BioPlex 2.0. Automatic loading of publications from PubMed, their abstracts, MeSH terms, and analysis of the frequency of occurrence of MeSH terms and protein names, as well as visualization of connections between them, were performed using the ScanBious web tool (https://scanbious.ru/).
Results: In our work we analyzed the terms describing protein functions used in neXtProt and monitored how and why the “profile” of the functional diversity of the human proteome has changed in recent years. The “look into the past” of functional annotation allowed us to evaluate the readiness of the proteomic community for the transition from a description of the functions of genes to a description of the functions of specific proteoforms (Paik et al., 2018). Potential experimental approaches for functional characterization of uPE1 proteins can be divided into two workflows, PPI-based function prediction, and Multi-Omics Knowledge-based function prediction. The prediction results can further be tested by using Phenotypic Cell-based Screens that can utilize biochemical or immunologic assays for verification of the prediction results. These experiments can be used in a flexible manner as appropriate to verify and validate the function(s) of target dark proteins.
Conclusions: Our work accumulates history of existing computational, experimental approaches used for protein functional prediction and approvement.
Acknowledgments
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P11.24

Introducing a Cloud Scalable Omics Data Analysis Pipeline with a Serverless Task Infrastructure for Large Scale Proteomics Studies

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[1]*Seer*

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) has grown into a ubiquitous detection platform due to its speed, sensitivity, and applications. While instrumentation hardware continues to improve, the concurrent increase in translation from data to insight remains a bottleneck. Most computational proteomics pipelines are built for desktop environments and are not cloud-native or easily leveraged in distributed computing environments. A high level of scalability could be achieved by containerizing legacy applications and orchestrating them in cloud environments. In this work, we present an automated cloud-connected data processing solution for analyzing outputs from a fleet of MS instruments from multiple vendors, generating terabyte-scale data annually.

Our scalable platform begins with Watchdog monitors that transfer MS raw files, as they arrive, from our instruments into AWS S3 file storage. These trigger Lambda Functions, which act as connective tissue to Step Functions, which map out tasks, choices, and error-handling necessary for our analyses. Our Elastic Container Service Tasks, which accomplish our most computationally rigorous code, use Docker-containerized executables that are instantiated using a mixture of AWS's Fargate and Batch serverless paradigm. We leverage Batch when Fargate's compute and local storage is not sufficient, and Batch with Spot Instances for short but intense jobs to reduce costs. Our pipeline outputs are stored in a combination of S3 buckets, a non-relational Mongo database, and a relational PostgreSQL database, operating on a principle of polyglot persistence.

Seer's current database contains over 500 terabytes (and growing) of raw data from multiple MS vendors. Thousands of peptide/protein annotations are query-able using a polyglot persistence model of document and relational systems. Our pipeline utilizes AWS storage gateway services and automatically processes raw data. Users can also launch group analysis runs with pre-defined parameters. To date, thousands of analyses have been run on-demand and at scale.

P11.25

Increasing the Sensitivity of Neoantigen Identification in Mass Spectrometry-Based Immunopeptidomics Using Supervised Learning with Enhanced Peptide Features

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Introduction
The immunopeptidome is defined as the repertoire of peptides presented by major histocompatibility complex (MHC) molecules on the cell surface. Mass spectrometry (MS) is rapidly becoming an important tool in immunopeptidomics studies, but is limited by the non-tryptic nature of peptides, as well as their abundances, which are relatively low in the immunopeptidome compared to proteome digests. The computational identification from MS data therefore remains an important challenge. In recent years, the ability to accurately predict the binding and presentation of specific peptide sequences by MHC molecules has dramatically increased. Such predictions are commonly used in the filtering of validated peptide identifications, but they have not yet been utilized in the validation itself. Hence, we present a novel approach, called MhcValidator, that leverages these presentation predictions to improve the sensitivity of peptide identification in MS-based immunopeptidomics experiments.

Methods
MhcValidator is built around a feed-forward neural network. Presentation predictions from MhcFlurry and NetMHCpan are combined with common peptide-spectrum match quality metrics from target-decoy database searches. This dataset is used to train a binary-classification model which predicts the likelihood of the peptide spectrum matches in the experiment being true positive identifications.

Results
MhcValidator increases the sensitivity of peptide identification in most immunopeptidomics experiments at all false discovery rates (FDR), with increases of up to 40% at a FDR of 1% when compared against Percolator. Unlike current immunopeptidomics pipelines, these peptide identifications do not rely on the establishment of arbitrary thresholds related to binding or presentation scores of the peptides. We also demonstrate that integrating presentation predictions as part of the validation step improves the reproducibility of peptide identifications.

Conclusions
This work will ultimately enhance the identification of low abundance clinically relevant peptide epitopes for the development of immunotherapeutic and vaccine strategies against autoimmunity, infectious diseases and cancer.

P11.26

Interactive Statistical and Functional Analysis of Phosphoproteomics Data with Phosphomatics

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Introduction
While mass spectrometry-based phosphoproteomics routinely detects and quantifies thousands of phosphorylated peptides, interpreting this data can be challenging. In part, this is because comprehensive analysis frequently involves utilising many different software packages, web sites and databases resulting in a complicated and iterative process that is both prohibitive for non-experts and cumbersome and time-consuming for experienced researchers. Here, we present a substantial expansion to our phosphoproteomics data analysis website - 'Phosphomatics' – that incorporates a suite of new tools and resources for statistical and functional analysis that aim to simplify the process of extracting meaningful insights from experimental results.

Methods
Phosphomatics can natively import data from major search engines including MaxQuant and provides intuitive 'wizards' to guide users through common data preprocessing routines such as normalization and transformation. The website is divided into 4 main components for statistical, network, kinase and substrate analyses. Phosphomatics has been made publicly available and requires no programming experience to use.

Results
Following data upload, users are presented with a graphical platform of interactive univariate and multivariate analysis features that allows subgroups of the uploaded data containing phosphosites of statistical interest to be created and interrogated through further functional analysis. For example, features are incorporated to assess hyperactivation of putative upstream kinases, consensus phosphorylation motifs for peptides groups, pathway/gene ontology enrichment and interaction networks. A range of databases have been integrated that, for example, provide ligand and inhibitor information for key proteins or highlight key modification sites known to be involved in functional state regulation. At each step, published literature is natively incorporated along with a 'bibliography builder' that allows references of interest to be assembled and exported in various formats. Taken together, these expanded features aim to provide a 'one-stop-shop' for phosphoproteomics data analysis.

Conclusion
Phosphomatics is freely available via the internet at: https://phosphomatics.com/

P11.27

A Computational Tool for Comprehensive Selection of Potential Cancer Protein Biomarkers in Blood Plasma

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Introduction. Liquid biopsy has become an important tool for cancer screening, diagnostics, and monitoring, which requires high quality biomarkers detectable in blood. Among different types of biomarker candidates, proteins have attracted much attention because they are the nanomachines of life and can provide the pathological information at the functional level. Due to the large number of the proteins existing in blood, it is often necessary to pre-select potential protein markers before experimental studies. However, to date there is a lack of automated method for in-silico selection of cancer blood proteins that integrates the information from both genetic and proteomic studies.

Methods. In this work, we propose a bioinformatic pipeline taking advantages of major public databases in both genomics and proteomics for the identification of potential blood plasma protein biomarkers overexpressed in cancer. Here we demonstrated this pipeline with an example of human breast cancer. The Plasma Protein Database and several sub-datasets from the Human Protein Atlas were used to collect blood plasma proteins. Overexpressed proteins in breast cancer tissues were then selected. A set of overexpressed mRNAs in breast cancer that encode plasma proteins was obtained using GTEx and TCGA databases. The overlap between the overexpressed protein data and the mRNA data was shortlisted.

Results. We have identified 27 potential blood protein biomarkers for human breast cancer. All these 27 markers have been found to be linked with breast cancer in literature, which validated the usability of our technology.

Conclusions. To our knowledge this is the first computational tool for selecting plasma proteins based on key genomic and proteomic databases for cancer research. It provides a useful and efficient tool for the selection of cancer blood protein markers for a variety of cancer research ranging from basic cancer biology to diagnostics and treatment, accelerating the biomarker discovery and validation process.

P11.28

Implementing Comet Search Engine into Proteome Discoverer to Improve TMT Real-Time Search Data Processing

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Introduction
Real-time search (RTS) using Comet on the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer has enabled selective triggering of SPS MS3 scans upon confident identifications from MS2 spectra. Here we introduce the implementation of Comet in PD 3.0 to provide the best alignment between online and post-acquisition data analysis.
Method
For RTS experiments, 500ng Thermo ScientificTM PierceTM TMT11plex Yeast Digest Standard or TMT pro 18 plex HeLa sample was analyzed by an Orbitrap Eclipse Tribrid mass spectrometer (ICSW 3.5). MS2 spectra were searched against an yeast proteome database during acquisition using the Comet search algorithm (2019.01 rev.1The data were analyzed with Thermo ScientificTM Proteome DiscovererTM Software 3.0, using both Comet and SequestHT in combination with multiple PSM validation nodes for comparison.
Result
In both SequestHT and Comet in PD, parameters such as missed cleavages, variable and static modification, and mass tolerance were matched to the RTS Comet settings. Percolator, fixed value PSM validator and target decoy PSM validator were combined with each search algorithm to find the best alignment with RTS Comet search result. For data analysis without FDR criteria, the combination of PD Comet and fixed value PSM validator gave the closest alignment to the online database search result. 98% of PSMs that were confirmed by RTS Comet search were confidently identified in the post-acquisition analysis using PD Comet. If an FDR threshold is desired in data analysis PD Comet coupled with Percolator produced a higher number of identifications and better alignment with RTS Comet result. By combining both SequestHT and Comet in PD processing workflow, the number of identification and quantified IDs can be improved by 10-15%.
Conclusion
This study introduced the post-acquisition data analysis optimization using Comet node in PD 3.0 which has largely improved the data analysis of RTS experiments.

P11.29

AI Assisted Protein Identification and de Novo Sequencing in the Cloud

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Introduction
There is a pressing need for proteomics mass-spectrometry analysis software that scales well and is easy to use. Mass spectrometry datasets and protein sequence databases are both growing rapidly, as is the ubiquity of proteomics. Yet MS/MS analysis software is often slow and requires expertise to set the search parameters correctly. We present novor.cloud, a Google-like search solution that enables users to simply submit data for analysis and obtain more peptide sequences significantly faster, without the need to know all parameters a priori.

Methods
In earlier work, Rapid Novor developed software that can de novo sequence 300 spectra per second on a laptop and a database search algorithm that leverages de novo peptide sequence results to enhance mass-based database search. We now introduce a cloud-hosted web application to facilitate analysis. Novor.cloud further enhances speed and automatically determines the correct search settings (such as PTMs and error tolerance).

To test the effectiveness of this strategy we tested novor.cloud's speed, sensitivity, and automatic parameter setting.

Results
An 88-minute gradient of a HeLa lysate trypsin digest producing 59,223 MS/MS spectra was analyzed by novor.cloud and another search engine, MSFragger, on the same hardware. novor.cloud completed the search 3X faster and identified 5% more acceptable peptides. Even after activating 41 PTMs, novor.cloud completed the search 43% faster. Both search engines prove 30-100X faster than older database search algorithms.

To examine novor.cloud's ability to automatically determine the traditional search parameters, two searches were performed using the same data: one with expert human-guided parameters, one with no reasonable guidance given. Approximately 90% of the search results were identical.

Conclusion
Our approach can provide faster, more complete results and allows searches to be done easier without expert guidance on PTM selection and other search settings.

P11.30

lcmsWorld: High-Performance 3D Visualization Software for Mass Spectrometry

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Introduction
Complex biological samples in proteomics research are often analyzed using mass spectrometry paired with liquid chromatography or gas chromatography. The chromatography stage adds a third dimension (retention time) to the usual 2D mass spectrometry output (mass/charge, detected ion counts). Experimental results are often discovered by complex computational analysis, but it is not always possible to know if the data has been correctly interpreted. To perform quality-control checks, it can often be helpful to verify the results by manually examining the raw data, and it is typically easier to understand this in a graphical form.

Methods
3D graphics hardware is present in most modern computers but is rarely utilized by bioinformatics software, even when the data to be viewed are naturally 3D. lcmsWorld is new software that uses graphics hardware and native high-performance C++ programming to quickly and smoothly examine and compare LC-MS data.

Results
lcmsWorld allows a visual comparison across multiple files. It is easy, for example, to see the quality of replication between samples. Common problems such as chromatography contamination may also be seen by directly viewing the data across samples. Similarly, the presence of isotope patterns created by the uptake of labelled media (e.g., SILAC), are immediately obvious from viewing the data. The depth of identification coverage can quickly be seen with lcmsWorld, and individual identifications can be verified. Cases where an important identification occurred in one sample, but not in a similar sample, can be visually scrutinized. This can rule out other causes of the discrepancy, such as overlapping features causing missed identifications, and give more confidence in the observed result.

Conclusions
lcmsWorld provides a quick and easy way to view and visually compare LC-MS data. It is freely available as open source. Releases, source code, and example data files are available via https://github.com/PGB-LIV/lcmsWorld.

P11.31

MASH-Native: A Universal and Comprehensive Software for Native Mass Spectrometry and Top-down Proteomics

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Introduction
Native top-down mass spectrometry (MS)-based proteomics is a powerful method for comprehensive characterization of proteoforms and intact protein complexes in their native state. One of the challenges to practitioners of native MS is the complex datasets generated by native top-down MS experiments. Herein, we present MASH-Native, which provides various functionalities for native top-down MS data interpretation and processing by incorporation of UniDec, implementation of spectral averaging, and internal fragmentation searching. Importantly, MASH-Native is a free software and can process datasets from various vendors with multiple deconvolution and database searching algorithms.

Methods
MASH-Native is a multithreaded Windows application implemented in C# using the .NET framework. Data from various vendors are supported using ProteoWizard and other vendor-provided libraries. Processing workflows are supported by running algorithms with the appropriate input files and parameters and then parsing the results back into MASH-Native for interpretation. MASH-Native supports UniDec as a deconvolution algorithm by generating and parsing HDF5 files that are used by MetaUniDec. Internal fragmentation is also supported in MASH-Native. A GUI interface and processing workflow modifications were made to implement the spectra averaging feature.

Results
MASH-Native provides several tools to support native top-down MS data analysis. Importantly, MASH has incorporated the UniDec deconvolution algorithm into the suite of already available deconvolution algorithms. UniDec deconvolution may be run through either the MASH-Native GUI or using the UniDec GUI as selected in the MASH-Native deconvolution tab. The incorporation of new spectral averaging techniques allows users to choose how MS scans are averaged. To further increase protein sequence coverage in native and denatured proteoform characterization, internal fragment ion matching is also supported by MASH-Native.

Conclusions
MASH-Native provides many different avenues for processing native MS, MS/MS, and LC-MS/MS data. MASH-Native is a universal, comprehensive, user-friendly, and vital tool for any native or denatured top-down MS experiment.

P11.33

Deep Plasma Protein Characterization Enabled by Mass Spectrometry (MS) Data Acquisition and Machine Learning (ML) Methods.

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Introduction
Deep proteomic profiling of plasma using nanoparticles (NPs) has been previously described.1 Here we aim to further enhance the performance with addressing stochastic limitations of Data Dependent Acquisition (DDA) MS approaches. We model nanoparticle proteome profile and use real-time instrument programming to maximize the instrument duty cycle utilization and improve reproducible detection while maintaining the discovery characteristics of DDA methods. We further integrate deep learning approaches in peptide retention time prediction and precursor selection demonstrating improved identification rates and reproducibility of detection across injection replicates without compromising instrument's sampling rate.

Methods
Seer's Proteograph ProductTM Suite was used for sample preparation. Samples were run on a Pharmafluidics 50cm gen1-column with Thermo Fisher Scientific™ Orbitrap™ MS equipped with FAIMS Pro Interface. We leverage the application programming interface (iAPI) for real-time control of the MS acquisition, which allowed us to focus on historically detected peptides in plasma digests. Retention times were predicted using a deep learning approach. Downstream analysis was done using Proteome Discoverer 2.4 and FragPipe-v15.0, filtered at protein FDR (False Discovery Rate) level 0.01.

Results & Conclusions
The NP-specific peptide enrichment was modeled using the binomial distribution which showed >50% of peptides that we observed across several hundred historical runs show nanoparticle specificity and peptides that are enriched by a particular nanoparticle are consistently identified from run to run. Leveraging the NP-specific patterns of peptides, we made a hybrid method of targeted MS and non-targeted DDA that maximally leverages the instrument's duty cycle through an informed data acquisition. We then targeted up to 10,000 peptides in a standard DDA run and observe a resulting increase in peptide-spectrum matches, peptide and protein IDs using this method. This data indicating that informed MS may improve reproducibility and reduce stochasticity artifacts enabling more robust NP development.

References
1. Blume et al., 2020

P11.34

Improving the Sensitivity and Specificity of TMT-Labeled Phosphopeptide Identification Using Deep Learning

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Introduction
Tandem mass tag (TMT)-based tandem mass spectrometry (MS/MS) has become the method of choice for the quantification of post-translational modifications (PTMs) in complex mixtures. Many cancer proteogenomic studies have highlighted the importance of large-scale phosphopeptide quantification coupled with TMT labeling. Herein, we propose an in silico spectral library-based approach to improve both sensitivity and specificity in identifying MS/MS spectra of TMT-labeled phosphopeptides.

Methods
Both TMT labeling and phosphorylation can alter peptide fragmentation patterns during MS/MS, which makes the resulting MS/MS spectra of TMT-labeled phosphopeptides distinct from those of unlabeled, unmodified peptides, necessitating dedicated algorithms for improved identification. Deep learning enables us to figure out such complex fragmentation patterns. With deep learning-based fragment ion prediction, called DLPhor, we compiled an in silico spectral library of TMT-labeled phosphopeptides generated from ~8,000 human phosphoproteins annotated in Uniprot. The library consisted of 13,156,857 MS/MS spectra predicted by a deep learning model – (1) the annotated human phosphoproteins were (in silico) digested by trypsin allowing one missed cleavage; (2) STY-containing peptides were modified allowing up to two phosphorylation sites; (3) the resultant 4,385,619 unique peptides were ionized with charge states of 2+, 3+ and 4+ to generate predicted spectra.

Results and Conclusions
With DLPhor's library, we analyzed TMT-labeled phosphopeptide data from human-in-mouse xenograft breast tumor samples previously characterized by the Clinical Proteomic Tumor Analysis Consortium (CPTAC). The multi-stage strategy coupled with database search resulted in 10% increase in phosphopeptide identification. In addition, we discuss the target-decoy strategy in spectral library search. It is shown that the false discovery rate (FDR) was underestimated by the existing decoy spectra generation methods. We propose a new method to generate decoy spectra for accurate FDR estimation.

P11.35

"Oncoprogx": Innovative Proteogenomic Software Generating Sample-Specific Database for Mass Spectrometric Protein Identification

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Introduction: "Proteogenomics", a study integrating genomic and proteomic analyses, has significant benefits to identify gene products not discerned through genomic analyses alone. Mass spectra is utilized for identification of gene products in proteomics. Disappoitingly, mass spectrometric identification relies on the public protein database such as Swiss-Prot. Thus, the proteins with mutations unique to samples cannot be observed in the conventional mass spectrometric protein identification. To address this issue, our study aimed to develop a software generating sample-specific proteome database. We developed a novel software named OncoProGx. OncoProGx allows us to construct sample-specific proteome database with the data of whole exome and transcriptome. The purpose of this study is to evaluate the performance of OncoProGx.
Methods: The performance of OncoProGx was evaluated through comparison between OncoProGx and Swiss-Prot on two patient-derived sarcoma cell lines. Proteins, DNA, and RNA were extracted from those cell lines and subjected to masspectrometry and next generation sequence, respectively. Sample-specific database was generated from OncoProGx and used for mass spectrometric protein identification. The number and contents of peptides and proteins were compared between OncoProGx and Swiss-Prot. The identified peptides were also compared with single nucleotide variants based on the whole exome data.
Results: OncoProGx generated FAST files using the data of whole exome sequence and RNA-Seq. The contents of proteins identified by OncoProGx consisted of proteins derived from genetic alterations and splicing variants, which were unique to the cell lines and not recorded in Swiss-Prot. The number of the identified proteins were similar between OncoProGx and Swiss-Prot.
Conclusion: Because OncoProGx allows us to study intersample genomic heterogeneity, we believe that it enables us to offer important suggestions for biomarker development and target discovery.

P11.36

Expanding the Boundaries of Proteomics Data Integration and Visualization in Uniprot.

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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 20,000 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, Peptide Atlas and MaxQB.

Methods
Protein sequence data flows into UniProt through an established pipelines from databases such as the INSDC resources and PDBe, in addition to manual sequence submission by researchers using direct sequencing methodologies. Peptide data is then imported from collaborating databases such as PeptideAtlas and high-quality peptides are identified using well-defined quality metrics based on the work of the HPP, extracted by the pipeline and mapped to UniProt protein sequences. The effect of protein sequence import on protein representation and existence in UniProt will be analysed. Novel and evolving methods of proteomics peptide presentation in UniProt will be presented.

Result and conclusions
The import of protein sequence and proteomic data enriches the UniProt database, allowing it to act as a repository for an ever-expanding set of reference proteomics across the phylogenetic kingdoms. It is therefore of increasing importance that the UniProt team augment protein visualization and presentation of proteomics data, and look to use this information to increase our understanding of both protein expression and of post-translational modification status under different cellular environments.

All data are freely accessible from www.uniprot.org

P11.39

The Selection of Knockout Targets: HepG2 Multi-omics Profiling and Meta-Analysis

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Introduction
The totality of the same biological sample profiling results at the transcriptome, proteome, and metabolome levels provides a systemic understanding of the ongoing molecular processes and relationships between expression products in a gene-centric mode. The development of a multivariate digital portrait of the HepG2 cell line is an essential step in understanding the functional specificity of genes and the mechanisms of cell oncogenic transformation.

Methods
The cancer cell line HepG2 (SCC249, Sigma-Aldrich) was selected for the study. First, we prepared cell line samples under the unified conditions, after which we sent the cells for whole-genome and transcriptome sequencing (Illumina NovaSeq 6000 and MinION technology, Oxford Nanopore), proteome analysis (Orbitrap Fusion, Termo Fisher), metabolome profiling (LECO Pegasus 4DBT, Leco Corporation) and lipidome analysis (Maxis Impact qTOF, Bruker). The results of the experiments were compared with the current level of knowledge about the molecular characteristics of the HepG2 cell line, accumulated in scientific publications (for automatic topic-analysis of texts, the ScanBious module was used) and databases (Human Protein Atlas, Achilles, KEGG, MalaCards, DisGeNET, NeXtProt).

Results
We experimentally confirmed the expression of more than 13 thousand genes of the HepG2 cell line within this study, as well as 1.3 thousand protein products, 155 metabolites and more than 1,000 lipids. Integration of experimental data and information on metabolic pathways, protein-protein interactions, and data from cytogenetic studies made it possible to form a list of target genes for subsequent knockout and assessment of functional changes in the miltiome profile of the HepG2 line.

Conclusions
In the future, the accumulated data can provide the basis for creating predictive models for assessing the level of protein expression and identifying functional specificity, which are the priority tasks of the "Human Proteome" project.

This work was supported by the Russian Science Foundation (RSF Grant #20-14-00328; http://www.rscf.ru/).

P11.40

PTMeXchange: Reanalysis of Post-translational Modifications and Independent Estimation of False Localisation Rates

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Introduction

Post-translational modifications (PTMs) in proteins are widely studied using mass spectrometry (MS), which can provide data on the identification of types of PTM and localisation on particular protein sites. Scores and statistics have been incorporated to proteomics tools, giving an estimate of whether a site has been correctly identified but these are often calibrated using synthetic datasets and their statistical reliability on real datasets is largely unknown. This results in widespread reporting of incorrectly localised phosphosites due to inadequate statistical control. Within this project, we are reanalysing very large volumes of public PTM proteomics datasets, focusing on the main types of PTMs across different species, including human and model organisms (mouse, Arabidopsis), applying novel methods for control of global false localisation rate (FLR) for modification sites.

Methods

We have applied a concept of using decoy amino acids to allow for independent estimation of false localisation rates in phosphoproteomics datasets. We have tested and profiled several amino acids to act as a decoy, on both synthetic and real datasets, investigating the effect of decoy amino acid choice on FLR estimations.

Results

Amino acid selection can make a substantial difference to the estimated global FLR. Although several amino acids may be appropriate, the most reliable FLR results were achieved using alanine as a decoy, and for some cases with small database sizes, it was also appropriate to estimate global FLR using a statistical model derived from data distributions.

Conclusions

We propose that the phosphoproteomics field should adopt the decoy amino acid method for the estimation of FLR. This would result in better control over false reporting in the literature, and in public databases that re-distribute the data. We are developing analysis pipelines incorporating these methods for very large-scale re-analysis of public data sets, for broad distribution in the “PTMeXchange” project.

P11.41

RHybridFinder: An R Package to Process Immunopeptidomic Data for Putative Hybrid Peptide Discovery

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Introduction: Proteasomal spliced peptides (PSPs) are presented by Major Histocompatibility Complex (MHC) class I molecules and can trigger T cell responses in various disease contexts. Despite their demonstrated immunological relevance, their systematic identification by mass spectrometry (MS) is an unsolved challenge.
Methods: We used R and parallel computing to create RHybridFinder (RHF). The software tool is built upon the validated & published workflow by Faridi et al. (2018) for the discovery of hybrid peptides from PEAKS analyses. RHF also uses netMHCpan to enable quick analysis and summarization of peptide binding affinity to MHC molecules.
Results: Here, we present RHF, an R package for the systematic and relatively quick identification of putative PSPs by MS. To show its utility, we applied RHF on a dataset of 19 mouse tissues to show that PSPs could potentially represent up to 6% of the immunopeptidome across normal tissues. While the results are not yet validated experimentally, if validated, this would indicate that presentation of PSPs is a ubiquitous process.
Conclusion: RHF is available on CRAN: https://cran.r-project.org/web/packages/RHybridFinder/index.html Dissemination of this R package is a step forward to enabling more researchers to apply MS and explore those debated peptides. Furthermore, while RHF enables the computational identification of putative PSPs, experimental validation is required in order to confirm their source and presentation.

P11.42

SAPID-MSI: Spatially-Aware Protein Identification Algorithm for Mass Spectrometry Imaging

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Introduction

Mass spectrometry imaging is a technique that allows not only the identification of proteins in a sample, but also the determination of their localization within a tissue. Nevertheless, protein identification in mass spectrometry imaging is challenging, with many experiments only identifying hundreds of proteins in their samples. Thousands of mass spectra are typically acquired with mass spectrometry imaging, with a large number of them remaining uncharacterized due to their poor quality. In addition, most computational approaches for protein identification that are currently used in mass spectrometry imaging were originally designed for standard mass spectrometry-based proteomics and do not take advantage of the spatial information acquired in mass spectrometry imaging.

Methods

Herein, we introduce a novel supervised learning algorithm, SAPID-MSI, that integrates both spatial and local information acquired by mass spectrometry imaging to assess protein identifications at a given tissue location. Our approach is based on the idea that given some evidence that a protein P is present at a site S, the identification of P in the neighbourhood of S increases the confidence of P's identification at S. We used a combination of cross-validation and downsampling methods to assess the accuracy of SAPID-MSI's protein identifications.

Results

We benchmarked SAPID-MSI against ProteinProphet, a state-of-the-art tool for protein identification confidence assessment, using Piehowski et al.'s mass spectrometry imaging analysis of mouse uterine tissues. Our algorithm identified 10% more proteins than ProteinProphet at a 1% false discovery rate. We also show that SAPID-MSI detects significantly more proteins than ProteinProphet when less mass spectra are acquired at each site. SAPID-MSI's performances demonstrate that integrating spatial information in the protein identification process in mass spectrometry imaging improves its sensitivity.

Conclusions

Finally, by improving proteome characterization in mass spectrometry imaging, our approach will provide a better understanding of the processes taking place in biological tissues.

P11.43

Deep Learning Algorithm for CID Peptide Fragmentation Prediction

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Introduction: Collision induced dissociation (CID) is a historically used peptide fragmentation method for MS/MS. Recently, CID type instruments such as single/multiple reaction monitoring (SRM/MRM) MS/MS have gained more attention as a diagnostic tool for clinical Industries (1). However, novel algorithms for peptide fragmentation prediction mostly target HCD fragmentation. Here, we incorporated deep learning to develop a peptide fragmentation prediction algorithm specifically for triple quardrupole (QqQ-CID) type of data.
Methods: Datasets from NIST, PRIDE and laboratory synthesized peptides were formatted, parsed and filtered to form a final training dataset of 180,833 peptides (2, 3). Simultaneous to a RNN model development, the peptide fragmentation pattern from the dataset was analyzed to figure out the important features. The final algorithm's accuracy was than compared to previous studies.
Results: Analysis of peptide fragmentation pattern showed proline existence, peptide length and a sliding window of at least four amino acid combinations as important features during fragmentation. Along with RNN layers, these features were fed to the model in appropriate form and weights which increased the model's accuracy. The prediction accuracy of our model, PrAI-frag, exceeded the accuracy of other models that predicts CID type of fragmentation data.
Conclusions: PrAI-frag, predicts CID fragmentation spectra and is especially accurate for higher ranked fragments. This algorithm should benefit users of CID method instruments. Furthermore, it will be provided via a web server.
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P11.44

MetaProClust-MS1: An MS1 Profiling Approach to Metaproteome Screening

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Introduction: There is an increasing need to characterize how drugs may be altering the human gut microbiome and consequently affecting human health. Recently, metaproteomics has been used to explore the gut microbiome and its taxonomic and functional shifts when in the presence of drugs. However, acquiring metaproteomic data by tandem mass spectrometry (MS/MS) can be time consuming and resource intensive. To mediate this challenge, we present MetaProClust-MS1, a computational framework for rapid metaproteomic screening. This approach was developed to reduce the time required for MS data acquisition in drug-microbiome studies by using MS1-only profiling.
Methods: MetaProClust-MS1 first uses Independent Component Analysis (ICA) to remove noise introduced by MS1-only data acquisition. ICA is followed by a combination of K-medoid and hierarchical clustering to group drug treatments into clusters with similar effects on the gut microbiome. In a proof-of-concept study, we tested MetaProClust-MS1 on a gut microbiome sample treated with five different drugs that have known effects on gut microorganisms. These treated samples were analyzed using both an MS1-only and an MS/MS approach. Results of both data acquisition methods were compared to test the ability of an MS1-only approach to detect effects of drugs on microbiome metaproteome samples.
Results: We compared the clusters identified by the framework and found that MetaProClust-MS1 and the MS/MS analysis identified robust microbiome shifts caused by drugs. In addition, the drug treatment clusters detected by MetaProClust-MS1 and MS/MS share a high level of similarity ($r = 0.625$, p-value < 0.0001).
Conclusions: Metaproteome screening by MetaProClust-MS1 using MS1 profiles can detect metaproteome shifts upon treatments in a similar fashion as classic MS/MS approaches. However, data acquisition and resource requirements are drastically reduced when MS1-only profiles are used. MetaProClust-MS1 is intended to be an approach for data-guided high-throughput studies and will be especially useful for applications in personalized medicine.

P11.45

TIMS Viz for Mobility Offset Mass Aligned Interrogation of Complex Samples

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Introduction: The PASEF® acquisition mode of the timsTOF Pro has the power to isolate co-eluting, quasi-isobaric peptides separately for fragmentation, based on differences in the peptide's ion mobility. Such an event is called Mobility Offset Mass Aligned (MOMA) and results in non-chimeric spectra. TIMS Viz was introduced to visualize MOMA events in complex samples and was used to analyze data sets obtained from a whole cell lysate and phosphopeptide enriched sample.

Methods: Phosphorylated peptides were enriched using TiO2. Commercially available HeLa digest (Pierce) was used as representative cell lysate sample. All digests were separated on a nanoElute (Bruker Daltonics) coupled to a timsTOF Pro (Bruker Daltonics). Data analysis was performed using the real-time database search engine PaSER (Bruker Daltonics).

Results: TIMS Viz, a novel data visualization tool to display an interactive heatmap in the m/z ion mobility space, maps MOMA features. Herein, we show the number of MOMA groups, which are sets of at least two MOMA features, that could be identified by TIMS Viz with different m/z tolerance settings. Setting tolerances to 500 mDa and a retention time window of 10 s resulted in more than 40,000 MOMA groups containing more than 90,000 spectra for both, the cell lysate sample and the phosphopeptide enriched sample. Without the power of ion mobility separation these spectra would likely be chimeric in nature. Lowering the m/z tolerance to 25 mDa (well below the tolerance of any quadrupole) still leads to more than 18,000 MOMA groups (> 40,000 spectra) for the cell lysate and more than 23,000 MOMA groups (> 52,000 spectra) for the phosphopeptide enriched sample.

Conclusion: TIMS Viz helps user to explore their data for MOMA features and is a powerful demonstration how the TIMS dimension can improve the spectral quality for co-eluting, quasi-isobaric peptides.

P11.47

Glycan de Novo Sequencing by Deep Learning

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Introduction
Glycolysation is a common modification of proteins that is associated with several diseases and therapies. De novo sequencing of intact glycopeptides from MS/MS spectra can simultaneously determine the peptides, the glycolysation sites, and the glycans, without assisting databases.
Methods
GNN-Glycan constructs the glycan tree of an intact N-linked glycopeptide from root to leaves, starting from the peptide (root) and iteratively adding monosaccharides (leaves) to the tree. At each iteration, the model predicts the next monosaccharide based on the MS/MS spectrum and the partial tree obtained from the previous iteration. Each of nine monosaccharide classes is added to the partial tree to create a pool of candidate trees, then two neural networks are applied to select the best candidate. The first one captures the similarity between the MS/MS spectrum and the theoretical fragment ions of candidate trees. The second one, a graph neural network, captures the structure of candidate trees. Their outputs are combined to produce a probability distribution over the monosaccharide classes, from which the best monosaccharide and its corresponding tree are selected and fed to the next iteration.
Results
We trained and tested GNN-Glycan on a dataset of 23,608 glycopeptide-spectrum matches. The ground-truth glycopeptides were identified using PEAKS Glycan database search at 1% FDR. We compared the predicted de novo glycans to the ground-truth ones and calculated the accuracy at fragment ion level and glycan level. Our de novo sequencing model was able to identify 71% correct fragment ions and 41% correct glycans. We also found that the tree structure captured by the graph neural network substantially improved the prediction of de novo glycans, resulting in 26% more accurate glycans than using the fragment ions alone.
Conclusions
The tree structure of glycans can be learned by graph neural networks to improve glycan de novo sequencing.

P11.48

MS2ReScore: Using Predicted Fragment Ion Intensities and Retention Times to Increase Identification Rates in Metaproteomics without Impacting Sensitivity

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Introduction

The field of metaproteomics, the study of the collective proteome of whole (microbial) ecosystems, has seen substantial growth over the past few years. Despite its high relevance, the field still suffers from low identification rates in comparison to single-species proteomics. The underlying challenge here is a lack of sequence resolution and statistical validation in the current identification algorithms, which are typically designed for single-species proteomics [1,2].

Methods

To solve this issue, we updated and applied the machine learning-based MS²ReScore algorithm on several multi-species, metaproteomics datasets. In the original version of MS²ReScore [3], the search engine-dependent features of Percolator [4] were replaced with MS2 peak intensity features by comparing the PSM with the corresponding MS²PIP-predicted spectrum [5]. Here, we further improved the method by combining both feature sets and adding additional features from DeepLC [6], a novel deep learning retention time predictor. By combining all features, we gain enough sensitivity to drastically lower the estimated false discovery rate (FDR) threshold, while still retaining a higher number of identified spectra.

Results and Conclusion

When the updated MS²ReScore algorithm is applied on metaproteomics datasets, our results show that MS²ReScore leads to an increased identification rate, ranging from the number of PSMs to the taxonomic level, while the false discovery rate (FDR) remains under full control as validated in an entrapment experiment [7].

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P11.49

Identification of Murine Protein Homologs in the Chinese Hamster Proteome via Sequence Alignment and Machine Learning

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Introduction
The Chinese hamster ovary (CHO) cell is widely considered the most important cell line for production of biologics. However, compared to the mouse proteome, the CHO functional proteome remains poorly characterized, limiting our ability to engineer high-yielding cell lines. Homolog identification can aid in predicting the functions of CHO proteins that have not been studied experimentally.

Methods
We combined pairwise global sequence alignment and machine learning to identify the murine homologs of CHO proteins. To demonstrate proof-of-concept, this study was limited to phosphatases. We retrieved 544 and 986 phosphatases from the Chinese hamsters and mice proteomes, respectively. The cohort of all possible protein pairs was partitioned iteratively into 50 training and validation sets. By leveraging the alignment of known CHO-mouse homologs, we developed three random forest classifiers to discriminate between homologous and non-homologous proteins, one based on percentage sequence identity, another based on conserved protein domains, and the third based on both. These three models were trained on 50 training sets and subsequently evaluated on 50 validation sets.

Results
The identity-based classifier yielded an average sensitivity of 77% (±2%), specificity of 98% (±0.3%), accuracy of 98% (±0.3%), and area under the curve (AUC) of 90% (±0.9%) on the validation datasets. In contrast, the domain-based classifier yielded an average sensitivity of merely 37% (±2%) and AUC of 70% (±1.2%). This suggests that percentage sequence identity is more important than conserved protein domains in predicting protein homology. The third classifier, which included both sequence identity and protein domains as features, did not see a significant improvement in sensitivity or AUC compared to the identity-based classifier. This indicates that sequence identity alone is sufficient for achieving high sensitivity.

Conclusion
Our work improves the functional annotations of the CHO proteome and provides a computational framework for identifying homologous proteins in different organisms.

P11.50

Enhancement of MaCPepDB (Mass Centric Peptide Database)

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Introduction
Protein sequence databases, like the well known UniProt, provide basic information for most peptide identifications by proteomics search engines.
Based on UniProt’s sequence information MaCPepDB (https://macpepdb.mpc.rub.de/) was build, which contains the tryptic digestion of these sequences including the peptides’ masses.
MaCPepDB can help designing targeted proteomics experiments for single reaction monitoring and parallel reaction monitoring, where information about the peptide sequence, their masses and whether they are unique for one protein in a database or taxonomy is essential.
A web interface enables the user to search the information and refine it with additional filter parameters like mass tolerance, post translational modifications and taxonomy restriction.
With increasing number of users and peptides, MaCPepDB quickly reached the limit of its current single server architecture. Furthermore, improvements to the usability of the web interface were implemented.

Methods
To overcome the hardware limitations of a single server, the used database engine is replaced with a distributed engine. There are several suitable database candidates for a replacement, so the best one in terms of speed had to be validated.
The web interface is reimplemented to become more modular and more intuitive to use.

Results
The new database provides MaCPepDB with the ability to distribute the data and the workload on multiple servers and adapt to the increasing number of users and data, ensuring constant performance with respect to query times.
The improved web interface can help users designing their SRM/MRM or PRM experiments more quickly. Because of a higher modularity of the web interface, improvement suggested by our users can quickly be realized. With these adjustments e.g. a data export for Microsoft Excel was already integrated.

Conclusion
The implemented design changes make sure, that MaCPepDB is well suited for future UniProt releases increasing the amount of data and load due to more parallel users.

P11.51

The R-Package Prolfqua for Proteomics Label-Free Quantification Data Analysis

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We use prolfqua to develop highly customizable, visually appealing, and interactive data analysis reports in pdf or HTML format for quantification experiments. We use prolfqua to visualize and model simple experimental designs with a single explanatory variable and complex experiments with multiple factors. The prolfqua package integrates essential steps of the data analysis workflow: quality control, data normalization, protein aggregation, sample size estimation, modeling, and hypothesis testing. We further use prolfqua to benchmark data acquisition, data preprocessing or data modeling methods. We developed and improved the package by applying the "Eating your own dog food" principle, making it easy to use.

We use R's linear model and mixed model formula in prolfqua. R linear model and linear mixed effect models allow modeling parallel designs, repeated measurements, factorial designs, and many more. R's formula interface for linear models is flexible, widely used, and well documented. This approach makes it easy to reproduce an analysis performed with prolfqua in any other statistical programming language. We implemented features specific to high throughput experiments, such as the experimental Bayes variance and p-value moderation, which utilizes the parallel structure of the protein measurements and the analysis. We also compute probabilities of differential protein regulation based on peptide level models. Contrasts to test hypothesis can intuitively be specified in prolfqua using descriptive variable names, e.g., "Treatment_drug - Treatment_placebo".
The Benchmark functionality of prolfqua includes ROC curves and computes partial areas under those curves and other scores. We use it to study how well linear, mixed effect models or p-value moderation models quantitative mass spectrometric high throughput experiments.

Prolfqua is an easy-to-use R package to analyze quantitative mass spectrometric data and to report results. We used it to benchmark MS software and methods.

P11.52

CHIMERYS: An AI-Driven Leap Forward in Peptide Identification

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Introduction

Chimeric spectra are estimated to constitute >50% of DDA data, violating the assumption that one spectrum represents one peptide. Here, we describe Chimerys, a new search algorithm that rethinks the analysis of tandem mass spectra from the ground up. It routinely doubles the number of peptide identifications and reaches identification rates of >80%.

Methods

Chimerys uses accurate predictions of peptide fragment ion intensities and retention times provided by the deep learning framework Inferys. All candidates in the isolation window of a given tandem mass spectrum are considered simultaneously and compete for measured fragment ion intensity in one concerted step. Chimerys aims to explain as much measured intensity with as few candidate peptides as possible, resulting in the deconvolution of chimeric spectra. FDR-control is performed using Percolator. Chimerys is available through a node in Proteome Discoverer 3.0.

Results

Analyzing a HeLa tryptic digest (1 hour gradient) with Chimerys identified 114k PSMs, 61k unique peptides and 7,300 unique protein groups at 1% FDR. This is a 3.5-, 2- and 1.5-fold increase compared to SequestHT, respectively, resulting on average in 2.5-fold more identified peptides per protein (up to 30-fold in individual cases).
We successfully demonstrated the fidelity of Chimerys in four experiments: I) entrapment searches focusing on FDR-estimation, II) dilution experiments focusing on expected ratio distributions, III) comparisons with multiple search engines focusing on the overlap of identifications, IV) simulation experiments focusing on the deconvolution of chimeric spectra.
Chimerys is compatible with older mass spectrometer generations, but profits disproportionally from the increased sensitivity of recent instruments and measurements using wider isolation windows. It substantially outperformed SequestHT on data of different complexity such as body fluids and organisms from all kingdoms of life.

Conclusions

Chimerys is the first highly scalable, cloud-native, microservice-based and AI-powered search algorithm for the intensity-based deconvolution of chimeric spectra.

P12.A01

The HUPO-PSI Universal Spectrum Identifier (USI) For Mass Spectra

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Introduction

The availability of proteomics data in public repositories increased dramatically in recent years, and created the opportunity for researchers to inspect the mass spectrometry data of their peers. Unfortunately, this remained difficult because no standard mechanism was supported by these repositories. Therefore, the HUPO Proteomics Standards Initiative (HUPO-PSI) developed the Universal Spectrum Identifier (USI)[1], which provides a standardized format for referring to each publicly released spectrum from a dataset or 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, discussions, and shared documents we have developed a method of encoding a multi-part key that is effectively a virtual file path to each publicly released spectrum.

Results

The USI consists of the “mzspec” prefix, the collection component (PXD identifier), the mass spectrometry run component (e.g. name of the raw file), the index type (e.g. “scan”), the index number (e.g. the scan number), and an optional peptide interpretation. The USI has already been implemented by several repositories including PRIDE, MassIVE, jPOST, PeptideAtlas, and iProX. Moreover, the ProteomeCentral resource of ProteomeXchange implements a single endpoint that reaches out to the previously mentioned partners to fetch spectra for a provided USI (http://proteomecentral.proteomexchange.org/usi/). It also provides USI validator functionality, which checks that the format of the USI complies with the USI specification.

Conclusions

The USI provides a standardized mechanism for encoding a virtual path to any spectrum contained in datasets deposited to public repositories or contained in public spectral libraries. The USI will thus enable greater ease in communicating the spectra and interpretations of those spectra that are crucial as supporting evidence of scientific conclusions. The complete specification document is available at http://psidev.info/usi.

[1] https://doi.org/10.1038/s41592-021-01184-6

P12.A02

ProtView: A Software Tool for Protease Selection to Optimise Shotgun Proteomics and Investigate Transcript Activities

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Introduction: Trypsin is routinely used to digest proteins into peptides, despite not necessarily being the optimal protease for every type of experiment. Digest schemes with alternative proteases and their combinations have shown potential for increasing peptide identifications and protein sequence coverage in human, S. cerevisiae, E. coli, and A. thaliana, in addition to identifying regions not covered by tryptic peptides. In-silico software tools exist to evaluate digest schemes before carrying out analyses, mainly in a proteomic context. There is a need for a tool that can map theoretical peptides to both protein and genome references and allow protease evaluation for a wider range of analyses and biological questions.
Methodology: This poster presents ProtView, a versatile in-silico protease combination and digest evaluation workflow. It offers useful information in a proteomic and proteogenomic context, including protein sequence coverage, peptide length distributions, residue coverage, splice-junction coverage, genomic coordinates of peptides, and the number of unique peptides. A. thaliana data is used here to demonstrate the utility of ProtView and the outputs generated.
Results: ProtView can be used to optimise a broad selection of experimental aims, such as increasing proteome coverage, studying post-translational modifications that are associated with specific amino acid residues, and identifying alternative splice isoforms. Relative performances among digest schemes were correctly estimated by ProtView when benchmarked against published data.
Conclusions: ProtView presents information on digest schemes that can be used to optimise proteomic and proteogenomic experiments, saving on time, budget, and resources. ProtView is available at https://github.com/SSPuliasis/ProtView.

www.hupo2021.org
#HUPOreconnect
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P12.A03

PaSER Ex: Real Time Exclusion List

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Introduction:

Exclusion lists are powerful tools for increasing the number of peptides identified in a given sample. They contain peptide-specific measurements that the mass spectrometer is instructed to ignore. Here, we propose PaSER Ex, a dynamic exclusion list that can be updated in real-time.

Methods:

PaSER Ex extends the power of PaSER (Parallel Database Search Engine in Real-Time) by sending search results back to the mass spectrometer. We created an additional Kafka topic on the PaSER server for communication back to the instrument. There, identified peptides are stored in a 3-dimensional k-d tree, based on their mass, retention time, and ion mobility. To query the list, we utilized a range search with a +/- 100 second retention time window, +/- 20 ppm mass window, and a +/- 5% mobility window. During acquisition, all precursors are searched against the k-d tree and are only scheduled for fragmentation if there are no query results identified.

Results:

To test PaSER Ex, we performed a series of experiments with and without the exclusion list functionality. We found that experiments that utilized an exclusion list identified more unique peptides than experiments without. Furthermore, PaSER Ex had no significant effect on the instrument's duty cycle with an average query time of ~ 0.0001 for a list populated with 100,000 peptides. We plan to further investigate the benefits of using PaSER Ex on a series of identical samples, and how this compares to using a standard acquisition method with fractionated samples.

Conclusion:

PaSER Ex is one of the first implementations of bidirectional control for the timstofPro, which thus provides a foundation for the development of future bidirectional applications. It also eliminates the human error and time associated with searching data and generating exclusion lists.

P12.A05

Using Multilayer Heterogeneous Networks to Infer Functions of Phosphorylated Sites

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Introduction
Shotgun phosphoproteomics allows thousands of phosphorylated sites to be profiled in a single experiment; following data acquisition, researchers aim to ascertain differentially regulated sites that are functionally relevant to the biological processes being studied. Commonly used methods to analyse phosphoproteomics datasets depend on generic, gene-centric annotations such as Gene Ontology terms, which do not account for the function of a protein in a particular phosphorylation state. Currently, a lack of phosphorylated site-specific functional annotations hampers the analysis of phosphoproteomics datasets. We propose a method to infer the functions of phosphorylated sites from shotgun phosphoproteomics data, using a random walk on heterogeneous network algorithm.

Methods
Our approach has two key steps. First, a multilayer heterogeneous network is constructed by connecting phosphorylated sites from the dataset with the proteins they belong to, then the proteins to functional annotations. The second step is the application of a random walk algorithm, that ranks the nodes in the network; the higher the rank of the node representing a functional annotation, the more associated it is with a set of pre-selected phosphorylated sites. We optimised and assessed the performance of our method on a model dataset simulating the MAPK/ERK pathway, before assessing the performance on experimental shotgun phosphoproteomics datasets. We compared the performance of our approach against the current standard method, over representation analysis (ORA).

Results
We found that our method associated phosphorylated sites to their known functions in the model and experimental datasets. Fewer unrelated functional terms were found in the results compared to ORA, reducing the noise in the analysis. Random permutation of the network demonstrated that the ranking was driven by the network topology rather than chance.

Conclusions
Our approach provides a refinement of commonly used analysis methods and accurately predicts context-specific functions for sites with similar phosphorylation profiles.

P12.A06

Real-Time Selection of Glycopeptide Dissociation Methods by Matching Oxonium Patterns Using a Real-Time Library Search

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Tandem mass spectrometry (MS/MS) is the gold standard for intact glycopeptide identification, enabling sequence elucidation and site-specific localization of glycans. Beam-type collisional activation is generally sufficient for N-glycopeptides, while electron-driven dissociation is needed for site localization in O-glycopeptides. Modern glycoproteomic methods can utilize combinations of dissociation methods within the same acquisition, but sacrifices in sensitivity are often required when targeting more than one glycopeptide class. Oxonium ions are singly-charged fragments of mono- and polysaccharides, and differences in relative abundance between various oxonium ions are one spectral feature that has proven useful in glycopeptide identification and interpretation. In particular, specific oxonium ions can provide evidence for O-GalNAc or O-GlcNAc residues that indicate a spectrum belongs to either an N- or O-glycopeptide. Here we explore the utility of real-time library searching (RTLS) to match oxonium ion patterns for on-the-fly dissociation method selection. We investigate how modifying the newly released RTLS feature available on quadrupole-Orbitrap-linear ion trap Tribrid MS instruments can enable matching to library spectra comprised of pre-defined oxonium ion ratios. Originally designed for small molecule library spectral matching, RTLS is well suited to handle library matching to glycan fragments, and we use it to trigger sceHCD scans for matches to oxonium ion patterns of N-glycopeptide library entries while choosing EThcD scans for matches to oxonium ion patterns of O-glycopeptide library entries. This approach, termed Triggering via Oxonium ion Ratio Comparisons (TORC), enables equivalent numbers of N-glycopeptide identifications relative to standard sceHCD glycopeptide acquisitions while also generating comparable numbers of site-localized O-glycopeptide identifications from traditional EThcD-based methods. We also compare MS/MS success rates, non-glycopeptide identification rates, and various RTLS acquisition schemes. By matching dissociation method with glycopeptide class, both N-and O-glycopeptides can be site-specifically characterized within the same LC-MS/MS acquisition while maintaining sensitivity achieved when targeting each individually.

P12.A07

Unipept Desktop: Getting Unipept Ready for Proteogenomics

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Introduction
In recent years, proteomics has emerged as a novel technique that is situated at the interface of proteomics and genomics. It aims at improving the identification rate of proteomics data by first analysing metagenomes of a sample to better target the analysis of metaproteomes from the same sample. The genes predicted from a collection of reads are used to build a custom database for identifying measured spectra, instead of large general-purpose reference databases traditionally used in metaproteomics analyses. Limiting the protein search space improves sensitivity and specificity of metaproteomics analysis.

Methods
Unipept is a leading metaproteomics analysis tool that was initially developed as a web application. Its inherent web-based nature, however, limits the amount of data that can be analyzed. To overcome this limitation, we developed the Unipept Desktop application that is designed to drastically increase the throughput and capacity of data analysis.

Results and conclusions
The first stable version of the Unipept Desktop app was released in January 2021. It does not yet provide support for analyzing metaproteomics samples with custom databases, but focuses on improved analysis throughput.

Current development focuses on expanding the desktop app with support for custom databases. One way to reach this goal is providing an automated pipeline to filter Uniprot proteins for a given list of taxa and build a custom database that can be queried locally. As a result, no internet connection is required to query custom databases and bottlenecks caused by limited network bandwidth are no longer an issue. In a later stage, we would like to support true proteogenomics analysis and allow users to construct databases directly from an annotated collection of DNA reads.

P12.A08

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

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Introduction

Metaproteomics, the study of the collective proteome within a microbial ecosystem, has matured into a powerful tool to assess functional interactions in microbial communities. This maturation has been driven by improved technologies and informatics approaches[1] and by the realization that metaproteomics can provide functional insights into microbial communities that go well beyond what can be studied with other methods such as metagenomics[2]. Although a variety of metaproteomic workflows has been developed, their impact on the results remains to be established.

Methods

To evaluate and compare existing metaproteomic workflows, we carried out the first community-driven, multi-lab comparison in metaproteomics: the Critical Assessment of MetaProteome Investigation (CAMPI) study[3]. Based on well-established workflows, we evaluated the effect of sample preparation, mass spectrometry, and bioinformatic analysis using two samples: a simplified, lab-assembled human intestinal model and a human fecal sample.

Results

We found that meta-omics databases performed better than public reference databases across both samples. More importantly, even though larger differences were observed in identified spectra and unique peptide sequences, the different protein grouping strategies and the functional annotations provided similar results across the provided data sets from all laboratories. When minor differences could be observed, these were largely due to differences in wet-lab methods and partially to bioinformatic pipelines. Finally, for the taxonomic comparison, we found that overall profiles were similar between read-based methods and proteomics methods, with few exceptions.

Conclusion

To conclude, CAMPI demonstrates the robustness of present-day metaproteomics research, serves as a template for multi-lab studies in metaproteomics, and provides publicly available data sets for benchmarking future developments.

References
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P12.A09

Alignment Strategies of Dia Data and Their Effect on the Quantification Table

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Introduction:
Data Independent Acquisition (DIA) has become very popular recently for high-throughput proteomics using liquid chromatography coupled to mass-spectrometer (LC-MS/MS). One of the main advantages of DIA is its unbiased acquisition and reproducibility. However, as the size of a study increases, the problems related to chromatography start to appear more affecting the peptide quantification. In large-scale studies where data is acquired across multiple machines and even different geographical locations, the data matrix-completeness is severely hampered at 1% false discovery rate (FDR).

Methods:
I have developed a package, DIAlignR, which uses raw MS/MS chromatograms for alignment and reduces the number of false peaks in such studies. DIAlignR has multiple approaches for pairwise and multi-run alignment as per user's demand. For pairwise global, local and hybrid MS/MS chromatogram alignment are available. The pairwise approach is extended to multi-run alignment with star-based, rooted-tree based and unrooted-tree based methods.

Results:
On a manually curated 16-run dataset, DIAlignR reduces the FDR from 1% to 0.3% and increases the number of quantification events if FDR is extended to 1%.
To further test the performance, we used 227 runs acquired across 11 labs around the globe [1]. These HEK293 cell lysates + iRT peptides samples were spiked-in with 30 AQUA peptides in known concentration covering a dynamic range of five orders of magnitude. The coefficient-of-variation (CV) of AQUA peptide is significantly reduced after the alignment. The reduction is the most for hybrid pairwise approach extended with unrooted-tree based multirun alignment.

Conclusions:
We are presenting DIAlignR which improves the quality of data-matrix with fewer missing values. The method is tested on a manually annotated dataset and a large-scale heterogeneous 227 runs to rescue correct quantification events.

References:
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P12.A10

Real-Time Modification-Tolerant Matching of MS/MS Spectra at the Repository Scale

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Introduction:

How can the interpretation of newly-acquired tandem mass (MS/MS) spectra be informed by the billions of spectra acquired to date? This question is especially important for confirming the identification of surprising but important novel peptides/proteins, or for spectra that remain unidentified using standard methods. Furthermore, assessing the significance of novel identifications can benefit substantially from real time assessments of which tissues/datasets contain the same or modified/homolog versions of any peptide of interest. Conversely, repository-scale modification-tolerant matching is also an effective way to reject false positives by considering less-surprising interpretations of the same spectra as modified/homolog variants of otherwise commonly-detected peptides. We introduce a tool that enables these queries with near real time modification-tolerant searching against spectral libraries and public datasets.

Methods:

Repository-scale modification-tolerant searches are enabled using an indexing strategy and dynamic programming algorithms to determine the smallest portion of the search space to consider for a query spectrum without missing any true matches (at a given cosine threshold).

Results:

To create a repository-scale index, we used 13.8TB of spectrum files from 255 MassIVE proteomics datasets, consisting of 1.1 billion MS/MS spectra and associated identification information. Searching this index can be done using a web UI (massive.ucsd.edu) or on the command line (for offline/batch processing), allowing for repository search in ~14s for +200/-130 Da open search or 1.5s for a +200/-130 Da open search to the MassIVE-KB library (2.1M precursors) directly in a UI online using user spectra or public spectra USIs.

Conclusions:

Efficient indexing and algorithms enable real-time, modification-tolerant, repository-scale searches against billions of spectra enabling the use of full repositories to help confirm or reject novel identifications.

P12.A11

PRM-LIVE with Trapped Ion Mobility Spectrometry and Its Application in Selectivity Profiling of Kinase Inhibitors

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INTRODUCTION
Parallel reaction monitoring (PRM) has emerged as a popular approach for targeted protein quantification. With high ion utilization efficiency and first-in-class acquisition speed, the timsTOF Pro provides a powerful platform for PRM. However, chromatographic drift in peptide retention time (RT) represents a fundamental limitation for reproducible multiplexing of targets across PRM acquisitions. Here we develop PRM-LIVE a new acquisition scheme for the timsTOF Pro which dynamically adjusts detection windows to improve the multiplexing capacity of PRM. We demonstrate PRM-LIVE for activity-based selectivity profiling of small molecule kinase inhibitors.

METHODS
PRM-LIVE is implemented in Python integrated with the instrument API to monitor RT standards, calculate RT drift, and correct the scheduling window for each target. Competitive binding assays between kinase test inhibitors and multiplexed inhibitor beads as kinase activity probes were performed in human cell lysates, followed by kinase enrichment, trypsin digest, and PRM-LIVE analysis on the timsTOF Pro.

RESULTS
Using retention times for 300 HeLa tryptic peptides from a 60-min LC gradient as a reference, we demonstrated that PRM-LIVE could dynamically adjust the detection window for all peptides when using LC gradients of 45 min or 75 min. To test the maximum multiplexing, we scheduled 2014 HeLa peptides. PRM-LIVE captured ≥99% of the LC elution profile for 1857 of peptide targets, with 1736 of these exhibiting quantitation CV ≤ 20% across five replicate analyses. We next used PRM-LIVE to assess target selectivity across six reversible and covalent kinase inhibitors. Utilizing PRM-LIVE in our activity-based protein profiling assay we could reproducibly quantify inhibitor binding against 220 endogenous, cellular kinases. Our PRM-LIVE data confirmed the known inhibitor targets and discovered off-targets, which we validated in orthogonal assays.

CONCLUSIONS
Our new PRM-LIVE framework significantly improves multiplexing capacity for targeted proteomic analysis and is successfully applied in selectivity profiling of kinase inhibitors.

P12.A12

Mass Dynamics 1.0: Growing a Streamlined, Web-Based Environment for Analyzing, Sharing and Integrating Proteomics Data.

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Introduction
Mass Dynamics 1.0 (MD 1.0) is a web based analysis environment that can analyze and visualize bottom-up proteomics data. MD 1.0 utilizes cloud-based architecture to enable researchers to store data; enabling them to not only automatically process and visualize their proteomics data but annotate and share their findings with collaborators. Here we show how MD 1.0 is able to quantify complex proteomics data, and expand biological insights by leveraging existing knowledge bases such the GO ontologies and Reactome using pre-existing datasets..

Methods
MD 1.0 is composed of a javascript and rails app and open-source R packages utilized via Amazon Web Services (AWS) for a reproducible yet scalable workflow. We have evaluated MD 1.0 using well-characterised iPRG2015, dynamic range and HER2 datasets by comparison to existing platforms.

Results
MD 1.0 analysis of ground truth datasets were comparable to existing tools using both discrete and continuous measures. MD 1.0 architecture currently allows evaluation of various data inputs; from raw data to pre-processed data, such as MaxQuant output for LFQ and TMT datasets. MD 1.0 facilitates analysis, annotation and sharing of LFQ results and provides interactive and downloadable quality control reports, an automatic Reactome integration for Over Representation Analysis (ORA) and a GO/Reactome enrichment feature via the CAMERA algorithm from the LIMMA package. This is highlighted through re-analysis of existing LFQ studies, including the investigation of proteomic mechanisms for Her2 resistance.

Conclusion
MD 1.0 is a robust and reliable platform to analyse and share differential expression analysis and has the advantage of seamless integration with external public knowledge bases.

P12.B01

MS^3 Analysis of Glycopeptides Using MALDImini™-1 Compact MALDI Digital Ion Trap Mass Spectrometer

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Introduction: Glycans, which are one post-translational modification of proteins, are molecules with high structural heterogeneity which are formed by complex bonding of glucose, mannose, and other monosaccharides. It is known that their complex structure is related to regulation of protein functions, and various phenomenon can be observed depending on illness and other factors. Here, we present an innovative MALDI mass spectrometer that enables MS^3. Thus, this instrument enables insights into the glycan structure and into the peptide backbone.
Methods: The Glycoprotein was digested by trypsin. The recovered mixture of peptides and glycopeptides was separated by a SepharoseCL4B gel packed pipette tip and the glycopeptide fraction was overlaid with 2,5-DHB matrix solution and analysed with MALDImini-1™ (Shimadzu) compact MALDI mass spectrometer equipped with a digital ion trap (DIT).
Results: Plenty of different glycopeptides were observed in MS mode in the mass range between 2000 and 3500 m/z. In MS^2 mode, the glycan structure can be identified from characteristic fragment ion pattern. This analysis was followed by two MS^3 measurements. Firstly, a fragment ion without glycan sidechain was used as a precursor for the second fragmentation step to investigate the peptide backbone. Choosing a slightly heavier ion for the second fragmentation step enabled to determine the binding site of the glycan by comparing these two MS^3 spectra. MS^3 allows for deep insights into both the glycan structure as well as the peptide backbone of glycopeptides.
Conclusions: The results of this analysis show that the MALDImini-1™ compact MALDI-DIT mass spectrometer has a high MS^3 analysis capacity despite its small size and possesses the highest possible performance for obtaining full information for components like glycopeptides which have large molecular sizes and complex structures.

P12.B02

High Throughput Single-Shot Proteomics on the Timstof Pro 2

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Introduction
The timsTOF platform powered by PASEF technology has become a standard in proteomics enabling sensitive, accurate and robust proteomic analysis from a variety of samples. Recently introduced timsTOF Pro 2 instrument complements these benefits with improvements in ion transfer and robustness combined with optimized acquisition methods. Here, we show the performance of the instrument to quantify cell line proteomes in different gradients and discuss the best usage of the instrument for everyday use in biological experiments.

Methods
Digested HeLa peptides (Pierce, Thermo Fisher), digested K562 peptides (Promega) and inhouse digests from HEK cell lines were used for experiments. Peptides were loaded on a Aurora-25 cm column using nanoELute coupled to a timsTOF Pro 2 instrument. Data were acquired using DDA PASEF and dia-PASEF methods with different parameters tested for best performance. Data were directly streamed to PaSER box for all DDA data unless otherwise specified and processed offline in MaxQuant. DIA data were processed in Spectronaut. Data were filtered for FDR of 1% for peptide and protein groups.

Results
Initial experiments were performed with 200 ng of HeLa or K562 peptides and measured on 60-minute gradients. These runs typically resulted in identifying approximately 6000 protein groups. Under similar conditions, using inhouse digested HEK peptides with an improved sensitive sample preparation protocol, we could quantify approx. 7000 protein groups using DDA methods. With DIA analysis, HEK peptides resulted in about 8000 protein groups and more than 70000 unique peptide sequences. Furthermore, measuring 20 ng of peptides in relatively shorter gradients resulted in more than 3500 protein groups. We plan to further test other method parameters to arrive at new standard methods that could be readily applied by any user for high-throughput proteomics.

Conclusions
The timsTOF Pro 2 enables rapid and sensitive quantification of about 7000 protein groups in single-shot injections.

P12.B03

Doubly Functionalized Magnetic Microspheres with Immobilized Trypsin and LysC Enabling Fast, Easy and Automatable LC-MS Sample Preparation

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Introduction
Reproducible and efficient enzymatic digestion of proteins is one of the most important steps during proteomic sample preparation followed by LC-MS analysis.
We developed doubly functionalized magnetic microspheres coupled with LysC and trypsin, resulting in two advantages: high robustness and excellent enzymatic activity. We foresee fully automated high-throughput processes leading to highly purified peptides due to magnetic separation.

Methods
We tested the functionality and efficiency of the doubly functionalized enzyme beads with HeLa cells, yeast cells, single proteins and human plasma. We exchanged the in-solution digestion step of the iST workflow as followed: enzymatic digestion was performed using 100 µg of the doubly functionalized enzyme beads while shaking at 1400 rpm for 1 h at 37 °C and using 100 µg proteins of S.cerevisae or HeLa, 25 µg single protein or 2 µl of human plasma as sample. Subsequent clean-up following the iST workflow led to a total processing time of < 3 hours with less than 30 minutes hands-on time. LC-MS analyses were done on a TimsTOF Pro (45 min gradient).

Results
We first stress-tested the doubly functionalized enzyme beads for various conditions to check their stability. Surprisingly, they performed very well under harsh conditions such as highly reducing environment (50 mM Tris-(2-carboxyethyl)-phosphin)), high concentration of alkylation agents (200 mM 2-chloroacetamide), high temperature (60 °C) and extreme pH (5 to 11). For all tested conditions, almost no loss of function could be observed.
Additionally, we compared the doubly functionalized enzyme beads to commercially available trypsin beads and could identify 122 % more proteins and 233 % more peptides while reducing the number of missed lysine sites by 31 % using 0,6 OD600 of S.cerevisae as sample.

Conclusions
First-time ever two proteases coupled simultaneously to magnetic microspheres leading to robust, efficient, easy-to-use and automatable LC-MS sample preparation tools.

P12.B05

Deep Metaproteome Analysis using a Vanquish Neo UHPLC System Coupled to an Orbitrap Eclipse Tribrid with FAIMS Pro Interface

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Introduction
LCMS-based proteomics is a powerful tool for deep profiling of peptides and proteins in complex biological samples. Analyzing the proteome of microbial communities represents a challenge for current proteomics workflows due to the wide dynamic range of metaproteome. Extensive fractionation is required to address this challenge. Here we use gas-phase fractionation using the High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMSTM) coupled to a Thermo ScientificTM Orbitrap EclipseTM TribridTM mass spectrometer to maximize the proteome coverage.
Methods
1µg of peptides were separated using a Vanquish Neo nano-flow UHPLC system coupled to an Orbitrap Eclipse Tribrid MS with/without FAIMS. For the CV evaluation, eight CVs from -20 V to -90 V were used with a 60-minute gradient. Three of the CVs that showed the best proteome coverage and the least overlap in the peptides were selected. The final MS method was set to switch between different CVs with a top-speed method in a 3 second cycle time over a 140 minutes gradient.
Preliminary Results
The effect of gas-phase fractionation using the FAIMS was evaluated in a bottom-up proteomics setup. The raw files were searched against concatenated databases downloaded from UniPort using Thermofisher Scientific™ Proteome Discoverer™ 3.0 software utilizing, SEQUEST HT, and INFERYS re-scoring algorithm. Preliminary results showed over 10,000 proteins and 70,000 peptides in the ZymoBIOMICS Microbial Community standard for the No-FAIMS dataset. The addition of FAIMS improved protein identifications by about 19%. Similar improvement was observed in the ZymoBIOMIC Gut Microbiome standard dataset with identification of over 11,000 proteins and 80,000 peptides in the No-FAIMS experiment with a 17.5% improvement in protein identification when FAIMS is used.
Conclusions
Using the FAIMS for gas-phase fractionation of metaproteome improves the dynamic range coverage and limits the addition of more variability.

P12.B06

Rethink Tissue Lysis: High-Throughput Tissue Lysis Workflow Using the 'BeatBox' Platform for in-Depth Proteomic Coverage

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Introduction: Efficient protein extraction is a crucial and challenging step in tissue sample preparation. Physical disruption is typically used to homogenize rigid tissue structures and make proteins accessible for further processing steps. Conventional methods are faced with various challenges such as cross-contamination or low throughput.
Here we present a tissue lysis workflow on the newly developed 'BeatBox' platform enabling efficient protein extraction for 96 samples in parallel in 10 minutes.
Methods: The described workflow is based on 96 well sample processing on the 'BeatBox' platform and applicable for a plethora of tissue types ranging from soft brain to rigid heart muscle samples. Utilizing innovative magnet-driven technologies, a defined energy input is applied to each sample facilitating highly efficient protein extraction. For complete LC-MS sample preparation, we combined the 'BeatBox´-based tissue lysis with the iST workflow. Improved proteomic depth could be achieved by a 3-step peptide fractionation.
Results: From intact tissue sample to finished DDA data acquisition on a timsTOF Pro (Bruker) under 4 hours, we achieved around 2500 protein identifications for mouse heart muscle, around 3000 protein IDs for mouse liver tissue and around 4000 protein IDs for mouse lung tissue. At the same time, excellent digestion efficiency (> 85 % of peptides with no missed cleavage) and reproducibility (Pearson correlations of 0.96) were obtained. Using a combination of the ´BeatBox' workflow with tip-based peptide fractionation, we increased the number of protein identifications by over 40 % compared to unfractionated samples.
Conclusion: The innovative 'BeatBox'-based workflow will set a new standard in tissue sample preparation by enabling ultra-fast and highly efficient protein extraction in a high-throughput manner.

P12.B08

Evaluation of Dia-PASEF Using Library and Library Free Approaches for Different Gradients

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Introduction
dia-PASEF (Meier et.al.,2019) takes advantage of the additional dimension of separation provided by trapped ion mobility for the analysis of complex proteomics samples by data independent analysis (DIA). Here, we evaluate benefits of dia-PASEF comparing very short and long gradients. Moreover, we compared results from two independent software platforms which can process native dia-PASEF data using spectral libraries or a library-free approach.
Methods
K562 tryptic digest (Promega) was analyzed by coupling EVOSEP One (EVOSEP) or nanoElute (Bruker) system to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro 2). Data were acquired in DDA-PASEF mode and dia-PASEF using gradient lengths of 300, 200, 100, 60, 50, 30 and 15 samples per day (SPD). dia-PASEF schemes optimized for the short and long gradients covering m/z range from 400-1000 and distinct mobility ranges. DDA-PASEF data were processed with PASER (Bruker). Spectronaut 15 (Biognosys) and DIA-NN 1.8 (Demichev et.al, 2021) were used for DIA data processing either using both spectral library and library-free approaches.
Results
We created a K562 spectral library containing 8,018 PG (protein groups) and 116,870 peptides sequences using Spectronaut software. Using this library, we identified 7,100 PG / 97,000 peptides from 15 SPD method in dia-PASEF mode using Spectronaut 15. Additionally, we identified 6,866 PG / 102,000 peptides using DIA-NN 1.8 software and the same library. Using a library-free approach, we identified 6300 PG / 70,000 peptides using Spectronaut 15 and 7300 PG / 117,000 peptides using DIA-NN. DDA-PASEF analysis for the same gradient length returned 5,600 PG and 49,000 peptides. 3300 PG / 23,000 peptides were identified from a 300 SPD method using the spectral library while 3600 PG / 23,500 peptides with the library-free approach. DDA-PASEF acquisition mode for the same gradient identified 1300 PG / 5,300 peptides.

P12.B09

The Impact of a Plug and Play Microflow Ionization Source on High Throughput Proteomics

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Introduction: For high-throughput mass spectrometry; robustness, sensitivity and reproducibility are critical factors. The ProCan® Centre houses a suite of six SCIEX TripleTOFs 6600 systems running tens of thousands of cancer tissue biopsies and cell lines in DIA mode. They operate 24/7, requiring reliability and consistency to achieve maximum up-time with minimum maintenance. A source of reproducibility and instrument wear is sample ionization by electrospray ionization. These TripleTOFs were originally fitted with a Turbo V ion source. We aimed to determine whether a new plug-and-play microflow source could improve data reproducibility and instrument up-time, while decreasing cleaning frequency.
Methods: The Turbo V was compared with the new OptiFlow Turbo V sources which house pre-optimised probes and electrodes removing any need for adjustment. This was run in microflow mode, across all six instruments over six months, where the instruments were largely run 24/7 interspersed with various unfractionated tumor samples. A single bulk tryptic digest from a HEK cell line lysate and SCIEX SWATH Acquisition Performance Kits were quality controls.
Results: The intra-instrument CVs decreased on the majority of instruments running OptiFlow, resulting in peak area CVs of ≤10% on all instruments accompanied by a small increase in peptides identified on all but one instrument. The SWATH performance kit revealed a similarity of total proteins and peptides quantified between instruments. This demonstrates that source tuning is not a major source of results variance in an expert lab. Instrument up-time for six months pre and post OptiFlow increased across all but one instrument, with the largest gain of 18%, equating to approximately a month of additional run-time.
Conclusions: Overall the OptiFlow source improved data quality and consistency and enabled higher throughput. This provides significant benefit for single instrument laboratories, with even greater impact on the rate of throughput in a multi-instrument environment.

P12.B10

Exploring Human Brain Proteome with Alzheimer's Disease (AD) With MALDI Imaging Mass Spectrometry in Combination with Shotgun Proteomics

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Neuropathology of Alzheimer's disease (AD) is characterized by the accumulation and aggregation of Amyloid β (Aβ) peptides into extracellular plaques of the brain. Aβ is deposited not only in cerebral parenchyma but also in leptomeningeal and cerebral vessel walls, known as cerebral amyloid angiopathy (CAA). While a variety of Aβ peptides were identified, detailed production and distribution of individual Aβ peptides in pathological tissues of AD and CAA is not fully addressed. Here, we develop a novel protocol of MALDI-imaging mass spectrometry (MALDI-IMS) on human autopsy brain tissues to obtain a comprehensive proteoform mapping.
Human cortical specimens for IMS were obtained from those brains that were removed processed and placed in -80˚C within 8h postmortem at the Brain bank at Tokyo Metropolitan Institute of Gerontology. Frozen tissue sections were cut on a cryostat at a 10 µm thickness onto ITO glass slides. Spectra were acquired using the rapifleX MALDI Tissuetyper and timsTOF fleX in positive linear mode, whereas ions were detected with spatial resolution of 50-70 µm. Shotgun Proteomics from serial sections of MALDI-IMS were attempted using timsTOF Pro with nanoElute system.
MALDI-IMS with rapifleX MALDI Tissuetyper demonstrated the detailed distributions of both Aβx-40 and Aβx-42 (x = 2, 4, 5, 6, 7, 8, 9, and 11pE) in AD accompanied with moderate CAA brain. Furthermore, MALDI-IMS with timsTOF flex detected shorter Aβ peptide, including Aβ1-29, Aβ10-40 and Aβx-42 (x = 3, 3p). As the next step, we have challenged to integrate in depth AD brain proteome with MALDI-IMS and a shotgun proteomics using intact and on tissue digestion technology. For those shotgun proteomics data, we have compared epitope preference of peptide sequences from identified proteins in human brains. A new protocol that combines MALDI-IMS and shotgun proteomics is useful for elucidating the pathology of AD brains.

P12.B11

Absolute Quantification of 500 Human Plasma Proteins in Colon Cancer Plasma Samples by Prm-PASEF

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Introduction

Last year we communicated a first evaluation of the newly introduced prm-PASEF approach, which allows multiplexing the acquisition of several targets in a single ion mobility event without compromising sensitivity. We are now applying this approach to the absolute quantitation of 500 blood proteins in colon cancer plasma samples.

Methods

The plasma sample cohort consisted in 10 patients affected by a colon cancer (adeno carcinoma) and 10 controls. Plasma samples were depleted with a Mars 14 depletion column (Agilent), digested with a trypsin protease and spiked with a mixture of 800 quantified synthetic peptides (PQ500, Biognosys). All samples and controls were separated by nano-HPLC (nanoElute, Bruker Daltonics) on a pulled emitter column (IonOpticks, Australia) using a 100 min gradient. Peptides were analyzed on a timsTOF Pro instrument (Bruker Daltonics) operated both in prm-PASEF and dia-PASEF modes. Data processing has been done with Spectronaut (Biognosys), MaxQuant and Skyline-daily.

Results

We evaluated the quantification performance of the prm-PASEF in depleted plasma samples by monitoring 370 precursors with a 20 min gradient LC separation. We obtained a median number of 15 data points per elution profile and limits of detection down to 5.5 amole/µl using quantified synthetic peptides as reference. We demonstrated accuracy over more than 3 orders of magnitude of peptides concentrations with a maximum error on the determination of 20%. The median relative standard deviation of the signal of the peptides was of 3%. To increase further the peak capacity of the system, we are now using a 100 min gradient with a 25cm chromatography column packed with 1.6 µm particles and we monitored 1566 peptides precursors per prm-PASEF acquisition.

Conclusions

The prm-PASEF approach has been successfully applied to the analysis of colon cancer plasma samples. Finally, we will compare these quantitation results to those obtained using a dia-PASEF approach.

P12.B12

Comparative Evaluation of a New Processing Pipeline for Pasef Label-Free Quantification Analysis.

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Introduction
Parallel Accumulation Serial Fragmentation (PASEF) data acquisition strategies have changed the way proteomics data are recorded, and processed in many different way: the additional separation of target ions in the ion mobility dimension as well as the determination of their collisional cross-section (CCS) has increased the data files information content. using this supplementary information allows to increase identification reliability, data completeness, and quantitation accuracy. Here we have evaluated the performances of a newly introduced processing pipeline and compared it to the established MaxQuant and Peaks Studio platforms.

Methods
Tryptic digests from human cell line, yeast and E.Coli have been mixed in two different ratios. Each sample has been injected as quadruplicates on a nanocolumn (IonOptiks) with a 60 min gradient using a nano-LC system coupled to a timsTOF PRO mass spectrometer (Bruker). Data have been processed for label-free quantification using MaxQuant 2.0.1.0 (Cox Lab), Peaks X the (BSI) or Mascot Distiller 2.8 (Matrix Science).

Results
The Peaks X+ processing allowed to identify more than 9000 protein groups for the three proteomes, 7834 of which could meet the quantitation. 102 of the 4250 human proteins were measured with a ratio that was below 0,5 or above 2. For most of those proteins, a Skyline trace extraction allowed to underline that the correct ratio could be extracted from the raw data. A similar evaluation has been done using MaxQuant 1.16. Out of the 8562 identified, protein groups, 7840 could be quantified and only 2% of the Human proteins were having ratios below 0,5 or above 2. The correct ratio could again be inferred from a Skyline analysis

Conclusion
The communication will compare the results obtained from the same dataset while using the Distiller 2.8 pipeline and the latest versions of the Peaks and MaxQuant pipelines.

P12.B15

Impact of Improved MS/MS Duty Cycle On Protein Identification Efficiency using Data Independent Acquisition On a New QTOF Platform

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Introduction
Data independent acquisition (DIA) approaches have been shown to surpass data dependent acquisition (DDA) methodologies in terms of protein identifications in complex matrices especially at shorter acquisition speeds. A new QTOF platform equipped with a novel Zeno trap delivers MS/MS sensitivity gains (5-20x) in variable window SWATH acquisition due to duty cycle at MS/MS level exceeding 90%. Increases in protein and peptide identifications using Zeno SWATH vs. SWATH acquisitions at various sample throughputs were evaluated.
Methods
A research QTOF system was coupled to an EvoSep One EV-1000 (EvoSep) LC was acquiring at 30 SPD (30 samples per day, 44 min gradient), 60 SPD (21 min gradient) 100 SPD (11 min gradient), 200 SPD (5 min gradient) throughputs with 25, 50, 200 and 500 ng HeLa (Thermo Fisher Scientific) peptide loads injected in triplicate. SWATH acquisition and Zeno SWATH methods with variable isolation windows covered precursor mass ranges of 400-750 or 400-900 m/z. Data was processed with DIA-NN.
Results
Analysis of the same sample loads with Zeno SWATH rather than SWATH acquisition shows 50-140% and 80-130% increase in protein group identifications with CV <20% and CV <10%, respectively, at low (25-50 ng) peptide loads at different SPD throughputs. Precursor identifications with CV <20% and CV <10%, increase by 230% and 300%, respectively, with Zeno SWATH. At higher peptide loads (200-500 ng), with Zeno trap enabled, protein groups with <20% and <10% CV increase by 30-50% and 40-80%, respectively. At 500 ng HeLa peptide load with 30 SPD method, we are able to identify over 8400 protein groups and over 7700 protein groups in library-free mode with 89% and 95% of respective identifications having a CV <20%.
Conclusion
Zeno SWATH relative to SWATH acquisition substantially improves number of identifications, with ~90% of identifications having a CV <20%.

P12.B16

Benchmark of Micro-flow Chromatograph for Robust Proteomics Analysis

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Introduction
Liquid chromatography-mass spectrometry (LC-MS) has been a powerful analytical tool in protein identification and quantification. In the past few decades, nano-flow LC-MS has been the primary approach due to its high sensitivity. However, challenges always come from the needs of high throughput, reproducibility and robustness. Here we present a micro-flow LC-MS workflow using a robust setup with Thermo ScientificTM NG micro-flow UHPLC System coupled to Thermo ScientificTM Orbitrap ExplorisTM 240 mass spectrometer.
Methods
Thermo ScientificTM PierceTM HeLa protein digest standard and Thermo ScientificTM PierceTM TMT-11plex Yeast digest standard were analyzed on the Orbitrap Exploris 240 MS for LFQ analysis. Digested peripheral blood mononuclear cells (PBMCs) from a variety of animal species (human, mouse, rat, etc) were analyzed to demonstrate the robustness over 100 injections. Thermo ScientificTM High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) Pro Duo interface was installed to provide gas phase fractionation. Data was analyzed on Thermo ScientificTM Proteome DiscovererTM 3.0 software using MSPepSearch and CHIMERYS in parallel.
Result
We were able to identify ~3400 protein groups and ~27500 peptide groups from 1ug of HeLa digest, ~3800 protein groups and ~34000 peptide groups from 5ug of HeLa digest within 30min gradient. The micro-flow LC-MS system showed excellent reproducibility of protein group IDs (<3% coefficient of variation, CV) and protein group abundance (median CV <11%). In multiplex quantitation 90% of identified proteins and peptides were successfully quantified. Digested PBMC peptides were fractioned by 6 compensation voltages using FAIMS Pro Duo interface and were acquired in triplicate on the Orbitrap Exploris 240 MS. GPF provides deep proteome profiling without the need for off-line RPLC fractionation, which reduced the overall experiment time.
Conclusion
This micro-flow LC-MS setup has been demonstrated to be highly reproducible and robust without sacrificing performance for both discovery and quantitation. This study is for research use only.

P12.B18

Rapid and Reproducible Phosphoenrichment Using Fe-NTA Magnetic Beads

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Introduction
Phosphorylation is a critical post-translational modification that modulates the function of numerous proteins and recent advances in the mass spectrometry (MS) instrumentation have enabled studying phosphorylation at proteomics scale in complex biological samples. However, due to low stoichiometry of the phosphorylation in biological samples, affinity based phosphopeptide enrichment from milligrams of digest is required for MS detection and quantification. Here, we introduce an agarose-based Fe-NTA magnetic beads for the phosphopeptide enrichment workflows including application on a Kingfisher™ Apex Magnetic Particle Processor for high throughput use.
Methods
Nocodazole treated HeLa S3 cells were processed using Thermo Scientific™ EasyPep™ Maxi MS Sample preparation kit. The magnetic Fe-NTA beads were incubated with the protein digests and then magnetically separated from the supernatant manually or through automation using Kingfisher Apex Magnetic Particle Processor for the phosphopeptide enrichment. Unbound peptides were washed during the washing step and the phosphopeptides were eluted with a basic buffer. Peptides were quantified and normalized using the Pierce™ Quantitative Colorimetric Peptide Assay prior to LC-MS analysis using a Thermo Scientific Orbitrap QExactive Plus mass spectrometer. Thermo Scientific Proteome Discoverer 2.4 software was used to localize the phosphorylation sites.
Results
Our optimized EasyPep chemistry combined with the large-scale format and subsequent phosphopeptide enrichment was completed in less than 6-7 hours. We have identified ~8000-9000 phospshopeptides with ~95% phosphospecificity with low CVs (<5%) using the magnetic beads. We have compared it to the existing resin workflows and observed identical performance in terms of phosphopeptide specificity and identification rates. We have also assessed the workflow on a Kingfisher Apex Magnetic Particle Processor which ensures reproducibility and eliminates the hands-on-challenges while handling a large number of samples.
Conclusions
We demonstrate that the automated platform enables the enrichment of phosphopeptides using Fe-NTA magnetic beads with a greater phosphopeptide specificity.

P12.B19

SureQuant Targeted Mass Spectrometry Standards and Assay Panel for Quantitative Analysis of Phosphorylated Proteins from Multiple Signaling Pathways

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Introduction
There is broad interest in quantifying dynamic protein phosphorylation states in cellular signaling pathways under different conditions. Enrichment is necessary for better detection of the low abundant phosphorylated proteins, and multiplexed quantitation reagents parallelize processing across a multitude of experimental conditions. We have combined EasyPep™ technology, phosphopeptide enrichment, validated multipathway AQUA™ heavy-labeled phosphopeptide standards, and SureQuant™ targeted MS to quantitate changes in phosphorylated protein abundance across multiple stimulated cell lines. This novel workflow enables targeted quantitation of biologically relevant phosphorylation sites with high accuracy, precision, and specificity.
Methods
Multiple cell lines were grown with different stimulation conditions before in-solution digestion using EasyPep Maxi MS sample prep kit. One milligram of each digest spiked with phosphopeptides standard was subjected to analysis using the Thermo Scientific™ Pierce™ Fe-NTA phosphopeptide enrichment kit. Discovery and targeted LC-MS/MS analysis were performed using Thermo Scientific Dionex nanoLC™ system or Thermo Scientific NG low-flow UHPLC system coupled to Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap or Thermo Scientific™ Orbitrap Exploris™ 480 or Orbitrap Eclipse™ Tribrid™ Mass Spectrometers. Data analysis was performed with Proteome Discoverer and Skyline software.
Results
We have developed a complete workflow solution for targeted phosphopeptide analysis by combining EasyPep MS sample prep kits and SureQuant MS assay. Our optimized workflow combines Fe-NTA enrichment with 131 AQUA heavy-isotope phosphopeptide standards to monitor multi-pathway signaling pathway proteins. Two targeted MS methods (PRM and SureQuant) were compared to assess the relative performance for quantitation of the desired endogenous peptides. More than 100 endogenous phosphopeptides from multiple stimulated cancer cell lines and all 131 heavy phosphopeptides were quantitated with high sensitivity and reproducibility. SureQuant method allowed quantitation of endogenous phosphopeptides at 10x lower levels than PRM.
Conclusions
SureQuant multipathway phosphopeptide standard with novel SureQuant MS analysis allows reproducible, routine, and simultaneous quantitation of functionally relevant phosphorylation sites.

P12.B20

Engineered Multi-Nanoparticle Panels Enable Unmatched Depth and Sensitivity in Plasma Proteomics in Combination with Trapped Ion Mobility Mass Spectrometry

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Blood plasma is a rich, readily available source of proteins that is commonly used in clinical profiling studies. However, proteome research is inherently constrained by the large dynamic range of proteins in plasma. The ability to overcome these hurdles while interrogating the plasma proteome deeply and broadly has only been partially addressed by laborious, low throughput and non-scalable workflow. Our recently introduced Proteograph™ Product Suite enables high-throughput in-depth plasma proteome quantification, employing a panel of five engineered nanoparticles (NPs) with distinct physicochemical properties. This panel of NPs is used in parallel to provide optimized identification of plasma proteins in terms of depth and breadth with precise quantification.

Here we explore the synergy of the Proteograph using a plasma pool from healthy individuals with the timsTOF Pro and timsTOF SCP mass spectrometers (MS). We have investigated short and long Liquid Chromatography (LC) gradients ranging from 7 to 90 min using both data-dependent- and data-independent-acquisition strategies (i.e., DDA and DIA) evaluating depth of proteome coverage, dynamic range, throughput, and precision of Proteograph proteome profiling platform. The high efficiency of ion-beam sampling facilitated by the novel ion optics upstream of trapped ion mobility cartridge of the timsTOF SCP increased sensitivity by about 5-fold compared to timsTOF Pro. The combination of timsTOF SCP with an optimized NP panel enabled us to quantify thousands of proteins in less than 30 min LC-MS/MS acquisition time from plasma at only 80 ng sample load. In summary, Proteograph Product Suite together with the timsTOF Pro and timsTOF SCP provide a high-performance combination workflow for rapid deep, and precise plasma proteome profiling for biomedical research and biomarker discovery.

P12.B22

Low Abundance Protein Detection after Acetone Precipitation Using the ProTrap XG

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Introduction
Sodium dodecyl sulphate is commonly used during protein extraction before mass spectrometry. Efficient removal of the detergent must be achieved to obtain clean data as residual detergent competes with peptides for ionization.
Methods
A standard protein mix of 3 purified proteins (beta-galactosidase (50 ug), cytochrome c (50 ng) and alpha-enolase (1ng)) was precipitated in 50 mM Tris pH 8.0, 50 mM NaCl in the presence or absence of 2% SDS in the ProTrap XG with four volumes of acetone, digested after reduction and alkylation. Digested peptides were desalted using the integrated SPE cartridge. Samples were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer using an EACY-nLC and a 60-minute gradient. Residual SDS level was measured with the MBAS assay. The effect of increased NaCl on acetone precipitation was explored by precipitation BSA in the presence of 0.5 to 5% SDS, with residual SDS measured by MBAS assay.
Results
Residual SDS level in the standard protein averaged 30.03 +/- 13.99 ppm. 150 ng of the mixture was analyzed, with all three proteins (4 orders of magnitude) positively identified. Coverage ranged from 90 to 60 %. To further determine limits of the salt effect, 50 µg samples of BSA were precipitated in the presence of 0.5 to 5% SDS with variable NaCl concentrations, increasing up to 300 mM NaCl. At higher SDS concentrations and 300 mM NaCl, increased variability in SDS removal was observed. Optimal results were obtained at 2% or lower SDS in the presence of 150 mM or lower NaCl. The use of the ProTrap XG decreases the variability observed during precipitation.
Conclusions
Precipitation can be used to reproducibly and reliably remove detergent contamination. Precipitation can be used with both abundant and rare proteins successfully. The ionic strength of the starting matrix influences precipitation reproducibility.

P12.B23

Combining the Data-Driven and Hypothesis-Driven Approaches in One Go via a Novel Intelligent Data Acquisition Hybrid-Dia Mass Spectrometry Strategy

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Translational scientists face the dilemma to choose between comprehensive profiling and sensitive targeted quantitation, especially with large sample cohorts. Proteomic profiling is commonly used to discover biomarkers, having a great potential for prognostic and predictive biomarkers; however, it still misses the sensitivity to quantify all the markers of interests. Therefore, targeted quantitation experiments of the potential markers are analyzed in the validation phase. This leads to high cost, time losses and more sample consumption. To address these challenges, we develop a novel intelligent data acquisition "Hybrid-DIA" MS strategy that enables comprehensive proteome profiling via high resolution MS1-based data-independent-acquisition (HRMS1-DIA) MS and on-the-fly intelligently switching the acquisition mode to parallel reaction monitoring (PRM) for sensitive quantification of the markers, substantially increasing throughput and reducing sample consumption.
The Hybrid-DIA strategy consists of a standard DIA scan cycle, where MS scan is followed by DIA MS/MS scans. Fast (multiplexed) PRM MS/MS scans are triggered from MS data based on isotope labelled peptides' signals and are used as a second layer of confirmation for isotope labelled peptides. Successful isotope labelled peptide detection triggers the high-quality measurement of corresponding endogenous peptides multiplexed (msx) with the isotope labeled peptides through msxPRM MS/MS scans acquired with narrower isolation window width and maximizing ion injection time for each species. The global profiling and quantitation performance of Hybrid-DIA MS are investigated and benchmarked against the standard DIA MS methods by analyzing mixes of stable isotope labelled peptides spiked in HELA and plasma digest, respectively. Similar number of proteins are identified with 1% FDR and quantified with CV<20% by both the Hybrid-DIA and DIA experiments; while Hybrid-DIA method can simultaneously quantify endogenous biomarkers with high precision and reproducibility.
This novel Hybrid-DIA MS methodology presents a new capability to combine the data-driven and hypothesis-driven approaches in one go.

P12.B24

Reproducibility and Sensitivity of a Targeted Quantitative Assay for 804 Peptides in Plasma Using a 20 Min Microflow Gradient

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[1]*SCIEX*

Introduction: Proteomic workflows cover a wide range depending on project goals, from fully untargeted data dependent acquisition (DDA) approaches to fully targeted quantitative assays for the highest specificity/sensitivity (MRM). This work investigates the impact of the Zeno trap for increased MS/MS sensitivity on peptide quantitation. The Zeno trap provides significant gains in peptide fragment signal by trapping ions in the Zeno trap region of the collision cell, then releasing them such that all ions arrive as a condensed packet at the same time in the TOF accelerator region.
Methods: The PQ500 kit (Biognosys, 804 heavy peptides) was used for peptide quantification testing. Microflow chromatography was performed on a Phenomenex Kinetex 150 x 0.3 mm LC column at 5 µL/min. A 20 min gradient was used, and concentration curves were generated for 2 amol-40 fmol on column in 500 ng of digested plasma. Samples were analyzed using the ZenoTOF 7600 system, and all experiments were performed with and without the Zeno trap activated. Data was processed using SCIEX OS software and Skyline.
Results: To test the impact on peptide MS/MS sensitivity, a targeted peptide quant assay was built using the PQ500 kit (804 peptides) and a 20 min gradient. With Zeno trapping implemented, the average gain in MS/MS fragment peak area was ~5.6 fold. Across ten replicate injections in plasma, the peak areas of the fragment ions were extracted and summed, giving a median peak area CV was 6.1%. The peptide areas were then evaluated across the concentration range for signal/noise, reproducibility and accuracy of the calibration curve. The median LLOQ was found to be 193 amol on column and the median LLOD was 114 amol on column.
Conclusions: A highly multiplex targeted peptide quantification assay has been developed to explore the quantitative capability of Zeno trapping.

P12.B25

Increased Protein and Peptide Identifications using Zeno MS/MS in Data Dependent Acquisition Workflows

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Introduction: In data dependent acquisition (DDA) workflows, the ability to collect high quality MS/MS at fast acquisition rates is key to maximizing peptide and protein identifications. Novel Zeno trap functionality can greatly improve duty cycle in the orthogonal pulsing region of a QTOF system, providing large gains in MS/MS sensitivity. This work investigates the impact of Zeno trap MS/MS sensitivity increases on protein and peptide identifications using microflow chromatography.

Methods: Digested K562 cell lysate was used in all DDA experiments with a range of sample loadings tested (25 ng – 2 µg). Microflow chromatography was performed on a Phenomenex Kinetex 150 x 0.3 mm LC column at 5 µL/min, with four linear gradients (5, 10, 20, and 45 min) were tested. Samples were analyzed using the ZenoTOF 7600 system, and all experiments were performed with and without the Zeno trap activated. Data was processed using OneOmics suite.

Results: For DDA experiments, acquisition parameters with Zeno trapping were first optimized for 4 different microflow gradient lengths using a design of experiments (DOE) approach. Using the optimized settings for 200 ng K562 digest, comparison data for Zeno trap on and off was generated for all gradients. Specific gains with Zeno on increase with gradient length, with gains in protein identifications of more than 35% for longer gradients. Sample loading curves were also generated at all gradient lengths to fully explore acquisition space, with more than 3000 proteins identified using a 45 min gradient with a 400 ng K562 load.

Conclusions: The Zeno trap results in increased protein and peptide identifications in DDA workflows. The gains achieved using the Zeno trap enable use of lower sample loadings for DDA analysis and improve the quality of libraries generated for SWATH acquisition data processing.

P12.C01

Can the Ultra-fast Proteomics Be Quantitative: Benchmarking directMS1 Method against Label-Based and Label-Free Approaches

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Introduction.
Recently, we introduced an MS/MS-free ultra-fast proteomic method, DirectMS1, allowing quantitative proteome characterization at the depth of more than 2000 proteins within a few minutes of experimental time (PMID: 32077687, 33720732). However, the question remains on the value of quantitation results obtained for the biologically relevant sample using this method in comparison with the approaches commonly used in proteomics.

Objectives.
The main objective was the evaluation of quantitation results obtained using DirectMS1 for the known biological model and their comparison with the standard label-free (LFQ) and TMT quantitation analyses.

Methods.
Glioblastoma cell lines were treated with type I interferon α-2b. Both treated and control samples in four biological replicates were prepared for DirectMS1, LFQ, and TMT quantitation analyses. Five-min LC gradients were used for DirectMS1 method. DDA-based LFQ results were obtained earlier (PXD022836) using 90-min LC gradients. TMT data were acquired in two ways: (1) single-shot 40-min LC-MS/MS run; and (2) samples were fractionated into 10 fractions followed by 60-min LC-MS/MS runs for each fraction. LFQ and TMT data were processed by Proteome Discoverer 2.5. Biosaur, ms1searchpy, and Diffacto were used for identification and quantitation of DirectMS1 data.

Results.
Comparison of the methods showed that ultra-fast proteomics based on DirectMS1 provides similar results with the other two: the same interferon-regulated signaling pathways were activated. While DirectMS1 method identifies and quantifies only 1500 to 2000 proteins, it performs as good as long-gradient LFQ and TMT methods (~4500 proteins) in revealing regulated proteins, yet, the latter two require more analysis time by factors 18 and 120, respectively. Further, DirectMS1 outperforms single-shot TMT method, which takes the same analysis time, in terms of quantitation results and the quality of the statistical analysis.

Conclusions.
Ultra-fast proteomic method DirectMS1 provides quantitative characterization of biological samples comparable with the long-run fractionation-based TMT and LFQ analysis.

P12.C02

SMART-CARE: A Systems Medicine Approach to Stratification of Cancer Recurrence Facilitated by Automated MS-Based Clinical Proteomics

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Introduction:
Mass spectrometry (MS)-based proteomic technologies gain momentum for molecular profiling of clinical specimens, to improve disease classification, diagnostics, and therapy development. Yet, hurdles need to be overcome to enhance reproducibility especially in large sample cohorts. Therefore, we here developed a streamlined workflow that integrates tissue lysis, protein clean-up by autoSP3, proteolysis, and LC-MS in an end-to-end and largely automated manner, for any sample type. We demonstrate proof of concept in the proteomic profiling of histologically defined pulmonary adenocarcinoma (ADC), and we will implement it more broadly to the stratification of cancer recurrence in SMART-CARE, an initiative to leverage LC-MS-based technologies for systems medicine.

Methods:
We combined AFA-based ultrasonication using a Covaris LE220R-plus with our single-pot solid-phase-enhanced sample preparation (SP3) method [1] on an Agilent Bravo system to establish a generic, end-to-end pipeline for concurrent processing of 96 cell-, tissue (fresh-frozen or FFPE), or liquid biopsy samples (autoSP3) [2].

Results:
To demonstrate the robustness of our autoSP3 workflow, we assessed its intra-day and longitudinal inter-day precision reaching Pearson correlation coefficients above 0.95. Reaching reproducible peptide and protein quantification starting from low input (down to 5 ng protein, or to 100 counted cells) further highlight the sensitivity of the workflow. Lastly, technically challenging and quantity-limited ADC (FFPE) growth patterns were successfully profiled, associating several signature proteins with tumor invasiveness.

Conclusion:
We established a generic, automated workflow comprising AFA-based ultrasonication and autoSP3 to enable routine, robust and comprehensive proteome analysis from any sample type, featuring minimal hands-on time, low sample consumption, low variability, high sensitivity, and longitudinal reproducibility. We will use this in a systems medicine-setting in the SMART-CARE initiative for the stratification of cancer recurrence.

References:
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[2] Mueller et al, Molecular Systems Biology 2020, 16(1): e9111.

P12.C03

Systematic Identification of ALK Substrates by Phosphoproteome and Interactome Analysis

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Introduction
There has been a remarkable improvement in the sensitivity of phosphorylation site identification by mass spectrometry. However, the lack of kinase-substrate relations (KSRs) information hampers the improvement of the range and accuracy of kinase activity prediction. In this study, we aimed to develop a method for acquiring systematic KSR information on anaplastic lymphoma kinase (ALK) using mass spectrometry and apply it to the prediction of kinase activity.
Methods
We established dox inducible ALK expression cells. Using our dox inducible ALK expression cells, we performed time-course phosphotyrosine proteome analysis. We identified 73 phosphotyrosine sites which were upregulated more than 1.5 fold in either 2, 4, 8 or 24 h after Dox induction . We also performed interactome analysis using formaldehyde-crosslinking. After crosslinking, Dox induced cells (Dox +) and control cells (Dox -) were lysed and ALK-interacted proteins were immunoprecipitated and quantified by label free quantitation (LFQ).
Results
We identified 732 protein groups which were significantly precipitated ($q < 0.05$). Finally, we selected 37 phosphotyrosine sites (22 protein groups) which were overlapped between up regulated phosphoproteome data and ALK-interactome data as candidates of ALK-substrate. 37 ALK substrate candidates were identified by integration of phosphoproteome and crosslinking interactome analysis of doxycycline inducible HEK-293 cell. Furthermore, KSRs of ALK were validated by in vitro kinase assay, phosphoproteome analysis of ALK gene mutated cell lines. Finally, we confirmed that the prediction of ALK kinase activity was improved by KSRs acquired in this study.
Conclusions
Our approach is applicable to other kinases, and future accumulation of KSRs will help us to estimate kinase activity and elucidate phosphorylation signals in cells more accurately.

P12.C05

Targeted UHPLC-MS/MS Proteomic Analysis Using QPrEST and Single Point Calibration with Application to the Determination of Apolipoproteins in Human Plasma.

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Introduction: Targeted Mass Spectrometry in combination with heavy isotope labeled protein standards are one of the most successful approaches for absolute quantification of proteins. The golden standard for the use of internal standards (IS), is the construction of external calibration with purified unlabeled standard proteins. This approach is time consuming and thwarted by the availability of pure, accurately quantified, unlabeled standard proteins. Herein, single point calibration was tested to quantify the targeted protein directly, using QPrEST standards as IS. The tested approach was applied to the determination of apolipoproteins in human plasma using UHPLC-MS/MS in PRM mode cross validated using ELISA.

Methods: Samples for testing the single point calibration approach against external calibration curve were all prepared by dilutions of IS-spiked human plasma in surrogate matrix (Chicken plasma). Plasma samples collected from 30 individuals were spiked with QPrEST standard mix. Spiking levels were relevant to the endogenous levels of the targeted proteins based on previous experiments. Samples were reduced, alkylated and digested with trypsin prior to the UHPLC-MS/MS analysis in PRM mode cross validated using ELISA.

Results: The results from the single point calibration were within 15 % difference from the results obtained from the external calibration curve method that was obtained by amino acid analysis. The same approach was applied to the determination of apolipoproteins in human plasma. Tryptic peptides of the investigated proteins have shown high intra-protein correlation and produced concentration levels within the reported endogenous levels. The results from UHPLC-MS/MS analysis were found correlated with the values obtained from ELISA of the target proteins, in the same plasma samples.

Conclusion: Single point calibration in combination with QPrEST standards provides a fast and highly correlated mean for absolute protein quantification applying UHPLC-MS/MS that is supported by cross validation using ELISA.

P12.C07

Analyzing Protein Fluorosequencing Data, a New Technology for Single Molecule Proteomics

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Introduction

Tools for protein identification and quantification lag DNA and RNA sequencing techniques in sensitivity and throughput, pushing proteomics researchers to pursue new high-performance approaches. To address these issues, our group invented fluorosequencing, a single molecule protein sequencing technology that incorporates features of nucleic acid sequencing for proteomics.

In fluorosequencing, proteins are proteolytically digested into peptides, and specific amino acids are labeled with fluorescent dyes. Labeled peptides are immobilized in a flow-cell where, using Edman degradation chemistry, they are sequenced in parallel while being imaged by single molecule microscopy. Fluorosequencing produces sequencing reads from many individual molecules simultaneously, with the expected elevation in noise and errors that must be addressed in subsequent computational analysis.

Methods

By modeling sequencing errors (failed chemistry, labeling, dye bleaching, etc) on large synthetic datasets, we considered four machine learning approaches to accurately assign fluorosequencing reads to parent peptides: (1) the k-Nearest-Neighbor method, (2) a Random Forest classifier, (3) Bayesian classification based on Hidden Markov Models of the chemical processes used in sequencing, and (4), a hybrid approach combining the k-Nearest-Neighbor method with the Hidden Markov Models.

Results

For small sets of proteins, RF, HMM, and kNN+HMM classifiers significantly outperformed kNN for classification precision and recall, with the HMM based classifier giving the best results. However, at human proteome scale, involving hundreds of thousands of possible peptides, RF and HMM models become intractable. We found that kNN+HMM offers a good compromise, scaling to the full proteome and significantly outperforming kNN.

Conclusions

A hybrid kNN+HMM machine learning strategy successfully assigns fluorosequences to their parent peptides at good precision and recall, while also scaling to the full human proteome and modeling known sources and rates of fluorosequencing errors in a human interpretable form.

P12.C09

New Method to Construct a Reference Amino Acid Sequence Database for Metaproteome Analysis

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Introduction: Metaproteomics using mass spectrometry is a powerful tool for profiling the vast number of microorganisms that inhabit humans such as gut bacteria. Since the early stage of metaproteomics, protein sequence databases have been actively constructed to identify peptides and proteins by large-scale human intestinal metagenome projects such as MetaHIT and HMP. The microbiome is so diverse, however, that it may not be covered by even the largest databases currently available. An organism with an unknown genome cannot naturally be identified because its protein sequence is also unknown. We have developed a method to probabilistically generate protein sequences of unknown organisms by using phylogenetic relationships among closely related species.
Method: A sequence set of 39 closely related species (Ther39) of Pyrococcus furiosus (Pfu) was classified into orthologous-like groups by homology search using blastp. Probability tables of substitution positions and amino acid differences were created for each group. After prior-analysis using the Ther39 sequence database, unknown species were located by inserting branches into the corresponding leaves of the Ther39 phylogenetic tree in the order of species with the most PSMs (effective leaf). Amino acid sequence for the unknown species was generated using the probability tables from the sequence given in the closest species. The LC/MS/MS data (PXD001077) downloaded from PRIDE was searched against the predicted sequence database, Ther39 and Pfu obtained from Uniprot using Comet (J. Am. Soc. Mass Spectrom. 2015;26:1865–1874) and Percolator (J. Proteome Res. 2009;8:3737–3745).
Results: The analysis using the effective leaf method yielded more target-peptides than the analysis using Ther39 which is not include Pfu. The coverage of both the PSMs to the Pfu results was improved by about 6%. These results suggest that the sequence generation of unknown species using sequence information of closely related species can improve the peptide identification rate of metaproteomics.

P12.C10

Label-Free Quantification of Oxidized Peptides in eHAP Cell Lines via a High-Throughput Dia-PASEF Workflow

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Introduction: dia-PASEF merges the benefits of data-independent acquisition (DIA) with the advantages of ion mobility in proteomics experiments. The ion mobility dimension improves the alignment of precursor and fragment spectra. In this study we applied a dia-PASEF workflow in combination with an Evosep One (Evosep) chromatographic system for high-throughput analysis of eHAP cell line digests, while quantifying spiked-in, oxidized target peptides in Spectronaut™ (Biognosys) on different gradient lengths.
Methods: The PreOmics NHS iST Kit was used to digest eHAP cells and 15 oxidized target peptides were spiked in at concentration levels corresponding to 0, 25 and 100 fmol/µg, respectively. Chromatographic separation was done using an Evosep One (Evosep) connected to a timsTOF Pro (Bruker) mass spectrometer. Standardized methods from the Evosep for 30, 60, 100 and 200 Samples Per Day (SPD) were used for library generation with a dda-PASEF method and the subsequent dia-PASEF measurements. Depending on the chromatographic method the sample loading varied between 20 and 200 ng. Data processing was done using Spectronaut (Biognosys).
Results: The number of protein groups in the spectral library generated from dda-PASEF data in Spectronaut (v14) ranged from 1,864 in the 200 SPD runs to 6,029 in the 30 SPD runs. The corresponding number of identified peptides were 8,605 and 39,947, respectively. In the subsequent dia-PASEF runs a recovery between 78.1 % and 97.8 % was reached in the 200 and 30 SPD runs, respectively. In the 30 SPD experiment 5,800 protein groups and 37,000 peptides were identified, whereas the faster 200 SPD experiment resulted in 1,500 protein groups with 6,000 peptides being identified. Additionally, the directDiaTM workflow in Spectronaut was compared to the library-based search.
Conclusions: In this study the best compromise between high throughput, sensitivity, and accuracy for the quantification of the spiked in target peptides were discovered for subsequent measurements.

P12.C11

Data-Independent Acquisition Method for Ubiquitinome Analysis Reveals Regulation of Circadian Biology

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Introduction:
Post-translational modifications (PTMs) are pivotal for the dynamic regulation of proteins. Among the vast set of PTMs, ubiquitination is one of the most studied PTMs and involved in a plethora of cellular processes. Given the mounting interest in analyzing ubiquitin signaling at a large scale we developed a workflow employing a state-of-the-art mass spectrometry (MS) method, data independent acquisition (DIA), to enable system-wide, in-depth ubiquitinome analysis.

Methods:
Our workflow combines antibody-based enrichment of peptides carrying a diGly remnant after tryptic digestion with an Orbitrap-based DIA method. We optimized instrument settings to tailor our DIA method for specific characteristics of diGly peptides and benchmarked this tailored method against conventional data dependent acquisition (DDA). To further increase the depth of diGly peptide analysis in a single MS run format, we constructed a modification-specific spectral library – containing over 90,000 ubiquitinated peptides – for matching.

Results:
This DIA-based approach allows the identification of 35,000 ubiquitination events in a single MS measurement from less than one mg of starting material. Compared to conventional label-free DDA strategies it doubles the depth of ubiquitinome analysis and reduces quantitative variation by 50%. Its application for system-wide investigation of ubiquitination across the circadian cycle uncovered hundreds of cycling ubiquitination sites and dozens of cycling ubiquitin clusters within individual membrane proteins such as receptors and transporters. These findings highlight novel connections between metabolism and circadian regulation.

Conclusions:
Our DIA-based workflow surpasses label-free DDA workflows and enables in depth, system-wide interrogation of the ubiquitinome of challenging biological systems, while requiring only small sample amounts. This pipeline can also be exploited for other PTMs relying on antibody-based enrichment. The single run format further makes it suitable for large-scale studies. We are currently exploring various chromatography/MS setups and automation of sample preparation steps for a more sensitive, streamlined and high-throughput workflow.

P12.C12

The Number of «Missing» Proteins Is a Function of the Analytical Sensitivity of Proteomic Analysis

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Introduction: To address the “MP-50”, the Russian part of the project evaluated the limits of the analytical sensitivity of the MS method on the example of UPS1 and UPS2 sets.
Methods: The analysis was performed using targeted mass spectrometry. The design of the experiment included the preparation of three types of samples: (a) a pure UPS1 solution (b) a solution with the E. coli matrix and pure UPS1 solution added in an equal ratio (c) a solution containing a constant concentration of E. coli matrix proteins and pure UPS1 solution.
Results: It has been found that 45, 44, and 25 proteins out of the 48 known to be present in the analyzed solutions were registered in the range of 10-9^10-10M, respectively. In the range of 10-10^10-11M, the number of identified proteins decreases to 28, 25, and 9. A further interval (10-12M) showed the possibility of using this method to detect only 10 out of 48 and 8 out of 48 proteins in solutions (a) and (b), respectively. With the reduction to the level of 10-13M, no proteins were detected from among the analyzed ones in any of the solutions.
To increase the concentration sensitivity, solutions (a-b) at a concentration of 10-12^10-13M were dried using a vacuum concentrator with the subsequent MS analysis of the samples. It was shown that after drying, the number of detected («recovered») proteins corresponds to the range of 10-9^10-10M and is, respectively, 90% of the proteins detected out of the 48 presents in the samples.
Conclusions: These results indicate the limits of application of targeted MS and difficulties of detecting "missing" proteins not associated with biological causes.

Acknowledgments
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P12.C13

Streamlined SDS-based Workflows in the ProTrap XG for Top-down or Bottom-up Proteomics

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INTRODUCTION
SDS is beneficial for front-end proteome sample preparation, though detrimental to MS analysis. Solvent precipitation is a proven approach to remove SDS ahead of analysis. However, achieving optimal protein recovery following solvent precipitation can be challenging. High yields are possible, assuming the proper conditions are met, including incorporation of sufficient ionic strength [1], as well as avoiding accidental pipetting of the sample pellet. The ProTrap XG is a disposable, two-stage filtration and extraction cartridge, designed to overcome the latter issue by isolating the protein pellet on a porous PTFE membrane [2]. Within this device, a complete SDS-based workflow for top-down and bottom-up sample processing is achievable.

METHODS
Standard proteins and yeast proteome extracts are spiked with SDS and subject to precipitation in the ProTrap XG. Proteins can be resolubilized and further purified with the integrated SPE cartridge ahead of TDP, or subject to in-cartridge digestion (trypsin, pepsin). Optimal protein precipitation and digestion conditions explore solvent composition (% organic, salt content, salt type) as well as time and temperature. Optimal digestion aims for complete (fully cleaved) proteolysis at minimal time using elevated temperature enhanced by the addition of calcium to preserve tryptic activity. SPE cleanups are integrated while multidimensional separation (SCX + RP) is also possible within the cartridge.

RESULTS
This presentation summarizes how high protein purity and recovery are essential for quantitative proteome analysis. The presence of residual SDS not only impacts protein separation and MS analysis, but compromises tryptic digestion efficiency as well. High efficiency digestion enables a rapid bottom-up workflow while formic acid enables effective MS-compatible resolubilization of intact proteins for TDP analysis.

CONCLUSIONS
The ProTrap XG streamlines SDS-based workflows for BUP and TDP analysis.

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P12.C14

Assessment of Bacterial Metaproteome Using Ultra-fast MS/MS-Free Proteomics

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Introduction: Metaproteomics is an emerging area of research to reveal the content and metabolic activity of microbial communities. It aims to identify and quantify proteins to differentiate community members, as well as characterize developmental stages of microbes, action of biotic and abiotic stress, changes in microbiome compositions and their functionalities (PMID: 19219053). Metaproteome analyses become feasible at the large scale with the advent of the high resolution MS (PMID: 31009573), yet, the long analysis time remains an issue. To facilitate microbiome studies, there is an urgent need for ultra-fast approaches allowing sample analyses in a few minutes. Here we present preliminary results on applying DirectMS1 method (PMID: 33720732) for characterization of bacterial samples.
Methods: Literature data was used to evaluate feasibility of our method to differentiate microbial organisms and their strains (PMID: 32998977). Biosaur (PMID: 33450063) and ms1searchpy (PMID: 33720732) were used for feature detection and protein identification in MS1 spectra. Experimental data were acquired using 5-min LC gradients and MS1-only mode for spectra acquisition. Model microbiome consisted of Rhodococcus opacus 1CP, Gordonia alkanivorans 135, and Priestia aryabhattai 25, mixed at different ratios.
Results: MS1-based analysis of 19 bacterial strains from the previous studies (PMID: 32998977) have shown that the sensitivity of the method allows differentiation of the strains of the same phylogenetic group. The efficiency of this differentiation is affected by the quality of the reference database used for protein identification. Another complicating factor is total database size that requires development of efficient data processing algorithms to shorten analysis time. We also found that bacterial proteome coverage correlates with a fraction of the strains in the total sample.
Conclusions: We demonstrate that the recently introduced DirectMS1 method of ultra-fast proteomics can be effectively used for in-depth characterization of microbial communities.

P12.C15

Number of Detected Proteins as the Function of the Sensitivity of Proteomic Technology in Human Liver Cells

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Introduction
The main goal of the Russian part of C-HPP is to detect and functionally annotate missing proteins (PE2-PE4) encoded by human chromosome 18. However, identifying such proteins in a complex biological mixture using mass spectrometry (MS)-based methods is difficult due to the insufficient sensitivity of proteomic analysis methods.
Methods
In this study, we determined the proteomic technology sensitivity using a standard set of UPS1 proteins as an example. The results revealed that 100% of proteins in a mixture could only be identified at a concentration of at least 10-9 M. The decrease in concentration leads to protein losses associated with technology sensitivity, and no UPS1 protein is detected at a concentration of 10-13 M. Therefore, two-dimensional fractionation of samples was applied to improve sensitivity. The human liver tissue was examined by selected reaction monitoring and shotgun methods of MS analysis using one-dimensional and two-dimensional fractionation to identify the proteins encoded by human chromosome 18.
Results
A total of 134 proteins encoded by human chromosome 18 were identified in human liver tissue samples.
Conclusions
The presented results indicate that the sensitivity of proteomic technologies is insufficient for the detection of all expressed proteins in liver cells. The solution to this problem is the concentration of biological samples, however, chromatographic columns have a limitation on the load in terms of the amount of protein, due to concentration of no more than 10 times is possible for 2D fractionation, which is clearly not enough, since it is not known in advance what concentration the proteins may be in the biological sample.

P12.C16

A Comprehensive Quality Control Pipeline for Clinical Biomarker Discovery

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Introduction: Increased speed and sensitivity of mass spectrometers enables automated workflows capable of delivering the high throughput required to support clinical proteomics. Despite that clinical biomarkers have been reported, their validation has been challenging. Contributing reasons for slow translation to the clinic have included irreproducible and poor-quality data in the discovery phase. The acquisition of high-quality data requires a strict quality control pipeline (QCP).
Methods: There is a suite of six instruments running in DIA mode 24/7 at ProCan®. The facility has acquired data from more than 60,000 runs across over 10,000 cancer biopsies and cell lines over the last four years. The sample cohort sizes ranged from 100-2,000 samples across time and between MS instruments. Data collection on this scale demands that quality is high and consistent across all samples, especially when one aim is to derive clinically valid assays. We have used this massive data set across time and space to develop and test a novel QCP.
Results: Samples are processed and data acquired in batches of 16, each including an instrument standard and sample preparation standard. A minimum of technical sample duplicates are run on different instruments for each sample. To assure high quality and reliable data, every data file is visually inspected for abnormalities in TIC, MS1/MS2 intensity and column pressure. Technical sample duplicates are overlayed to check that the results are comparable. A range of automated computational pipelines are used, including: AutoQC/LabKey software, monitoring a panel of 26 HEK peptides across all batches, and a first pass of each spectra though DIA-NN. A structured cleaning regime, based on defined quality thresholds, allows for maximum instrument reproducibility and uptime.
Conclusions: The data shows that the implemented QCP is extremely robust and illustrates how the ProCan® pipeline is suitable for high-throughput clinical applications in different laboratory configurations.

P12.C18

Comparison of Sample Preparation Methods and Instrumental Platforms for Proteomic Analysis of Murine Brain Tissues and Isolated Brain Cell Types

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Introduction
In parallel to continuous instrument improvements, major efforts have been recently invested in the development of innovative sample preparation methods dedicated to high throughput proteomics. The increasingly expanded proteomics toolbox today enables fine optimizations of each step of the workflow for any given sample type. In this context, we benchmarked performances of a classical stacking gel preparation against single pot solid-phase enhanced sample preparation (SP3) and in-stage tip digestion (iST, Preomics) on both total murine brain extracts and isolated brain cells. We also compared the proteome depth and coverage achieved on a Quadrupole-Time-of-Flight TimsTOF Pro instrument including an additional ion mobility separation against Quadrupole-Orbitrap instruments.
Methods
Total murine brain tissue extracts (MBT) were used to compare stacking gel preparations against iST digestion and murine brain isolated cells extracts (MBC) were used to compare SP3 against iST digestions. All peptide extracts were analyzed on either nanoLC-Q-Orbitrap instrument (nanoAcquity, Waters, coupled to a Q-exactive Plus or a Q-exactive HFX, Thermo Scientific) or on a nanoLC-TimsTOF Pro (nanoElute coupled to a TimsTOF Pro, Bruker Daltonics) platform.
Results
From MBT, 1,830 proteins versus 2,628 proteins were identified using stacking gel versus iST, respectively, while from MBC, 2,190 and 2,622 proteins were identified using SP3 and iST, respectively.
Using the highest performing iST digested samples, the proteome coverage was increased from 2,628 and 2,622 proteins to 4,797 and 4,065 proteins in MBT and MBC, respectively, using TimsTOF Pro.
Conclusion
Both iST and SP3 are promising alternatives to classical stacking gels and reveal to be complementary. In terms of coverage of the proteome reached, the TimsTOF Pro outperformed Q-Orbitrap instruments proving to be a potential workhorse for future proteomic experiments.

P12.C19

PEPPI-MS Workflow for Bottom-Up Proteomics

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Introduction: In mass spectrometry (MS)-based proteomics, sample fractionation prior to MS analysis can greatly contribute to improved results in protein identification and sequence coverage. To date, we have developed PEPPI-MS, a highly efficient recovery method for SDS-PAGE-separated in-gel proteins as intact species, and have established a sample pre-fractionation workflow for top-down proteomics [1]. In this study, we developed a sample preparation workflow for rapid enzymatic digestion of proteome fractions obtained by PEPPI and evaluated its effectiveness in bottom-up proteomics.

Methods: Reductively alkylated human cell lysates were separated by SDS-PAGE and stained with aqueous CBB. The sample lanes of the stained gel were fractionated and the proteins in the gel were recovered by PEPPI-MS. The recovered proteins were loaded onto a SAX-StageTip, and trypsin digestion was performed in the tip. Digested peptides were eluted from the discs with formic acid/acetonitrile solution and subjected to MS analysis.

Results: The PAGE-separated proteins were recovered in the CBB-bound state by PEPPI. When the recovered solution was loaded onto the SAX-StageTip, all proteins were captured on the top of the anion disk. Trypsin digestion in the tip (4 hours at 25°C) gave a digestion performance comparable to that of conventional in-gel digestion (18 hours at 37°C). Digested peptides were effectively recovered from the disc by formic acid/acetonitrile solution, but CBB and SDS, which interfere with MS analysis, were retained on the disc, avoiding their introduction into MS.

Conclusions: We have established a technique for rapid enzymatic digestion of PEPPI fractions using the SAX-StageTip, which enables sample preparation with minimal loss by completing the process from digestion to peptide purification within a single StageTip.

1. Takemori et al. 2020 J Proteome Res 19; 3779–3791

P12.C20

Specific Cysteine Sulfenic Acid Biomarker Screening by Coupling Mass Spectrometry with Laser Induced Dissociation Applied to Alzheimer's Disease and COVID-19.

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Introduction: In a context of population aging, discovery and validation of novel oxidative stress biomarkers of neurodegenerative diseases is a key issue. Oxidative stress could also be implied in COVID-19 infection. Reactive oxygen species induce, among others, protein cysteine thiol (SH) oxidation into sulphenic acid (SOH).[1] Thus, cysteine-SOH could be valuable candidates for biomarker screening in Alzheimer's Disease (AD) and COVID-19 pathways understanding. However, detection of these low abundance compounds is problematic due to the complexity and dynamic concentration range of biological samples.

Methods: To improve cysteine-SOH peptide detection specificity, we use a setup coupling mass spectrometry and laser induced dissociation (LID) at 473 nm, adding optical specificity to the mass selectivity. Specific photofragmentation is obtained by selective grafting of SOH groups with a Dabcyl cyclohexanedione chromophore.[2] To mitigate errors in inter-sample relative quantification of oxidation levels, the SOH/SH oxidative ratio is determined for each sample. For this, proteins SH were simultaneously grafted with a dabcyl maleimide chromophore.[3]
54 plasma samples (35 AD, 4 COVID-19, 15 Controls) were searched for cysteine oxidation, with top10 and PRM-LID methods covering 32 AD-related extracellular proteins, 15 plasma proteins and 1 COVID-related protein, totalizing 370 peptides

Results: Top10 analysis indicated an increase of oxidized α-antichymotrypsin (AACT) level in COVID-19 samples and CXCL7 (Platelet basic protein) over-oxidation in control samples. After PRM-LID investigation, cysteine oxidative ratios of AACT protein were slightly increased in COVID-19 samples compared to control samples, illustrating the role of ROS-related interactions during COVID-19 infections. CXCL7 oxidative level showed no correlation between samples. Only APOE oxidized protein was detected from AD-related biomarker screening, with no significant oxidative difference between control and diseased samples.

Conclusions: This novel methodology allows increased sensitivity of SOH detection with limited inter-samples analysis bias for large oxidative biomarker screening.

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P12.C21

Automated High Throughput DIA-MS Workflow for Plasma Proteomics with Novel Quality Control Procedure

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Introduction: There is a broad recognition that robust discovery workflows are essential to successful biomarker development pipelines and that a major challenge is balancing throughput, sensitivity, and reproducibility. Another concern for automated workflows is the quality control (QC) aspect of batch sample preparation. We developed a flexible workflow that allows for high and mid throughput analysis and reliable quantification of proteins in plasma and depleted plasma as well as an automated QC report for decision making on batch analysis.
Methods: Optimal conditions for sample preparation and DIA-MS analysis were established in plasma then automated and adapted for depleted plasma. The MS workflow was optimized for sensitive high-throughput or deep profile analysis with mid-throughput analysis. Analytical performance was evaluated from 5 complete workflows repeated over 3 days. Four QC samples were included in each batch of sample analysis spread in 4 different quadrants of a 96 well plate. QC samples were processed first and then analyzed automatically using OpenSwath and MapDIA pipelines, running on our in-house ProEpicTM software platform. Further automated analysis with pass or fail criteria was performed with an in-house developed script and emailed to the designated user at a specific time every day.
Results: Using our high-throughput workflow, 74%, 93% of peptides displayed an inter-day CV<30% in plasma and depleted plasma. While the mid-throughput workflow had 67%, 90% of peptides in plasma, and depleted plasma meeting the CV<30% standard. Lower limits of detection and quantitation were determined for observed proteins and peptides. Combining the analysis of both high-throughput plasma fractions exceeded the number of reliably identified proteins for individual biofluids in the mid-throughput workflows. Automated QC with pass or fail criteria has enabled quick decision on proceeding with a specific batch analysis or pausing for further investigation if there was a failure during automated sample preparation.

P12.C22

Critical Assessment of Salt Ions on the Recovery of Proteins through Solvent-Based Precipitation

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Introduction
Precipitation in organic solvent is a classic approach to purify and concentrate proteome samples ahead of mass spectrometry analysis. Our lab has shown that salt ions are essential to facilitate quantitative proteome precipitation [1]. The goal of the current study is to examine the precipitation efficiency of proteins in organic solvent (i.e. acetone), as influenced by the presence of different types of cations and anions. This work will lead to an optimized proteome purification and preconcentration protocol to maximize top-down and bottom-up proteoform characterization by mass spectrometry.

Methods
Bovine serum albumin and a yeast cell lysate act as model systems for the investigation. Proteins are precipitated in 80% organic solvent in the presence of varying types and concentrations of cationic and anionic species. Species selection will provide attention to classic (Hofmeister) chaotropic or kosmotropic species. Precipitated proteins are quantified via LC-UV, with assessment of proteins showing more favorable recovery via SDS PAGE and LC-MS/MS.

Results
A sigmoidal-shaped recovery curve is generated when precipitating proteins over a range of salt concentrations. While NaCl provides a sharp increase in recovery (<10 to >98%) between 1-10 mM, we have found that $ZnSO_4$ established similar yields at relatively lower concentrations (<1 mM). By contrast, $ZnCl_2$ requires a minimum 200 mM to recovery >95% of the protein. Na_2SO_4 is comparably less effective than NaCl. Thus, both cation and anion play important roles. Further investigation will establish if specific protein-salt interactions lead to precipitation.

Conclusions
The effects of different salts on protein precipitation in organic solvent were measured for the first time. This can guide the development of an optimal protocol for proteome sample preparation ahead of MS analysis.

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P12.C24

Enhanced Nano-Bio Interaction Enables Deep Plasma Proteomics at Scale, with Enhanced Precision, and Depths of Coverage.

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To overcome limitation of deep plasma proteomics in large cohorts, we have developed a fast and scalable technology that employs intricate protein-nano interactions. Introducing a nanoparticle (NP) into a biofluid such as blood plasma leads to the formation of a selective, specific, and reproducible protein corona at the nano-bio interface driven by the relationship between protein-NP affinity, protein abundance and protein-protein interactions. We previously demonstrated that this process, incorporated within the Seer Proteograph™ Product Suite, offers superior performance in terms of depth, breadth, precision, and throughput compared to conventional deep workflows. The ratio of plasma-to-nanoparticles determines the competition between proteins for binding surface, which plays an important role in protein corona composition and can be optimized to enhance and differentiate protein selectivity. Here we investigate effects of different conditions on protein corona composition enabling enhanced performance of Proteograph.

We have investigated compositional changes of protein coronas from 5 NPs with blood plasma at different ratios. Samples were analyzed with timsTOF Pro mass spectrometry and UltiMate3000 Dionex LC system using 30min DIA runs. We evaluated depth, dynamic range, coverage, and precision of quantification at a wide range of concentrations for each NP.

By limiting the available binding surface of NPs and increasing the binding competition, we are able to identify 20 – 60% more proteins on the surface of each NP. Moreover, by increasing the competition the proteins are more reproducibly identified and quantified across the replicates of the same NP. In addition, protein selectivity was enhanced, leading to improved coverage of plasma proteome when using multiple physicochemically distinct NPs. In summary, NP panels with optimized workflow, capture a large and diverse set of proteins and biological pathways based on their specific physicochemical makeup.

P12.C25

Neoantigens Identification and Personalized Vaccines Development from Immunopeptidomics Characterization

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Introduction
Immunopeptidomics is the science that studies the immunogenic peptides assembled in the Major Histocompatibility Complex (MHC), which activate T cells immune response. This complex list of immunopeptides is playing a high relevant role in precision medicine because it is directly related with individual genetic variation and susceptibility. Therefore, it plays a critical role in many diseases such as cancer, infections, inflammatory diseases, among auto-immune and neurodegenerative pathologies.

Methods
We made a systematic isolation of HLA molecules by specific and selective enrichment of HLA complexes by immuno-chromatography. The HLA complex is immunopurified from the cell lysate, and further elution of peptides from the captured HLA complexes, had been analyzed at high-resolution conditions, combining highly sensitive methodological approach by Data-independent acquisition (DIA) and Data-Dependent Acquisition (DDA) LC-MS/MS with computational biology which include the main databases used to identify potential peptides binding with these molecules.

Results
We anticipate our preliminary results obtained as a starting point for the identification of potential neoantigens from RAMOS tumour cell line (CRL-1596™ - Burkitts Lymphoma), as well as its in silico prediction that strengthens our study to move forward and the discussion of the next steps to be taken in their translation to the clinic.

In this sense, we identified 432 novo peptides with a length coinciding with the expected peptide presentation in HLA-I molecules, involved in Cell cycle checkpoints, HCMV, HIV and Influenza infections, ER-phagosome pathway, Antigen processing-cross presentation, Metabolism of proteins and Programmed cell death, among others.

Conclusions
In conclusion, the combined use of peptide identification and characterization techniques based on mass spectrometry, as well as their bioinformatics correlation with databases, may lead to new opportunities for personalized peptide vaccines targeting antigens against various pathologies such as cancer or infections, among others pathologies, and in this way allow to achieve a precision medicine.

P12.C27

The Isotopic AC-IP Tag Enables Multiplexed Proteome Quantification in Data-Independent Acquisition Mode

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Introduction: Data-independent acquisition (DIA) is an increasingly used approach for quantitative proteomics. However, most current isotope labeling strategies are not suitable for DIA, as they lead to more complex MS2 spectra or severe ratio distortion. As a result, DIA suffers from a lower throughput than data-dependent acquisition (DDA) due to a lower level of multiplexing.

Methods: We synthesized an isotopically labeled acetyl-isoleucine-proline (Ac-IP) tag for multiplexed quantification in DIA. Differentially labeled peptides have distinct precursor ions carrying the quantitative information but identical MS2 spectra, since the isotopically labeled Ac-Ile part leaves as a neutral loss upon collision-induced dissociation, while fragmentation of the peptide backbone generates regular fragment ions for identification. The Ac-IP labeled samples can be analyzed using general DIA LC-MS settings and the data obtained can be processed with established approaches. Relative quantification requires deconvolution of the isotope envelope of the respective precursor ions.

Results and conclusions: Suitability of the Ac-IP tag is demonstrated with a triplex-labeled yeast proteome spiked with bovine serum albumin (BSA) that was mixed at 10 : 5 : 1 ratios resulting in measured ratios of 9.7 : 5.3 : 1.1.

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P12.C28

FLASHIda: Intelligent Data Acquisition for Top-down Proteomics That Doubles Proteoform Identification Count

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Introduction
Top-down proteomics (TDP) is gaining great interest in biological, clinical, and medical sciences, as the method of choice to study proteoforms. While significant improvements have been made on different aspects of TDP protocols, data-dependent acquisition (DDA) has been optimized for bottom-up proteomics (BUP), not for TDP. Dedicated acquisition methods thus have the potential to greatly improve TDP. We present FLASHIda, an intelligent data acquisition method for TDP that ensures the selection of high-quality precursors of diverse proteoforms.
Methods
FLASHIda interfaces with Thermo Scientific iAPI that provides MS1 full scans real-time. By transforming the m/z-intensity spectrum to mass-quality spectrum instantly with FLASHDeconv and using a machine learning technique assessing the signal quality, FLASHIda implements Top-N high-quality precursor mass acquisition with a quality-based mass exclusion.
Results
In benchmark tests with E. coli lysate 90-min gradient single runs (nano-RPLC, Orbitrap Eclipse), FLASHIda almost doubled the unique proteoform count (~1,600) as compared with the standard acquisition (~820). Alternatively, similar numbers (~800) as with standard DDA were reported in FLASHIda runs on drastically shorter gradient runs (30-min). FLASHIda resulted in 20% more heavy proteoforms (>30 kDa) as well as one order of magnitude wider dynamic range than the standard. About 50% of the proteoforms from FLASHIda contained mass shifts, most of which corresponded to well-known modifications, e.g., methylation and oxidation. In particular, acetylated proteoforms were exclusively found in FLASHIda 90-min runs. We also found ~25% of the proteoforms were in truncated forms representing signal peptide cleavages or terminal degradations. In terms of the protein count, FLASHIda showed ~30% improvement upon the standard. Protein-level quantitative analysis results were highly consistent with those from previous E. coli BUP studies.
Conclusions
Since FLASHIda does not require major modification in experimental set-ups, it could be readily adopted for TDP study of complex samples to raise proteoform identification sensitivity.

P12.C29

Quantitative Assessment of Enzyme Activity in the Presence of Surfactants: Implications for Bottom-Up Proteomics

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Introduction
Proteome workflows rely heavily on denaturing surfactants to achieve quantitative sample recovery, affording comprehensive characterization of the biological sample. However, these surfactants are detrimental to chromatography and MS sensitivity. Bottom-up proteome approaches are equally dependent on a robust enzymatic digestion, which is most commonly achieved with trypsin, owing to its well-characterized specificity. In the interest of quantitation, digestion needs to be reproducible and complete, which demands sustained enzyme activity across the digestion period. The present work aims to characterize the effects of denaturing surfactants on the initial activity as well as the stability of trypsin over time to determine the optimal conditions for robust enzymatic digestion. Spectroscopic activity assays will be conducted in time course, followed by assessment of proteolysis efficiency in the presence of surfactants by LC-MS/MS.

Methods
TPCK-treated trypsin will be combined with a variety of denaturing surfactants (SDS, sodium deoxycholate, sodium laurate, guanidine HCl, CHAPS, etc.) and aged at 37 °C at pH 8.0 across a time course. Enzyme activity will be determined at each time point by a Nα-Benzoyl-L-Arginine Ethyl Ester assay. The conditions that show differences in activity will be used to digest samples of standard proteins and a proteome test sample (yeast or plasma). Digests will be subsequently analyzed by bottom-up LC-MS/MS to monitor the abundance of fully-cleaved peptides.

Results
Preliminary results show that the presence of low levels of denaturing surfactants (0.01% SDS, 0.2% SDC) can enhance the initial activity of trypsin, albeit temporarily. Even trace levels of these surfactants accelerate the loss in enzyme activity. However, the trypsin-stabilizing effects of calcium ions buffers the negative effect of surfactants on trypsin activity.

Conclusions
The presence of even trace levels of surfactants reduces trypsin activity. The subsequent bottom-up LC-MS/MS analysis will determine the implications of reduced enzyme activity and stability for proteome characterization.

P12.C30

Rapid Sample Preparation of Cancer Tissue Microarray Sections and FFPE Blocks for Clinical Analysis

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Many formalin-fixed paraffin embedded (FFPE) samples of cancer biopsies are available for proteomics. Among them, tissue microarrays (TMAs) are single paraffin blocks engineered from cylindrical tissue cores cut from multiple paraffin donor blocks and re-embedded together into a single TMA block of 100-1000 tumour samples. We aimed to develop a rapid and robust sample processing method with sufficient peptide yield for high-throughput MS-based proteomics using the smallest possible: a) thin sections from TMAs or b) narrow cylindrical cores.
The new proteomics workflow incorporated 'Heat and Beat' sample homogenisation and Pressure Cycling Technology. TMA sections were cut with a microtome at 10, 20 and 30 µm thickness. The average tryptic/Lys-C peptide yields were 1.0, 1.1 and 1.5 µg, respectively, sufficient for single MS runs with our high-throughput microflow MS platform, or for more sensitive deep proteome analysis.
To achieve multiple technical replicate runs, we applied the method to the smallest possible cores. Two core widths (0.6 and 1 mm), each with four core depths (0.25, 0.5, 1 and 2 mm) gave average peptide yields of 1, 2.9, 4.5, 4.5, 6. 9, 8.6, 11.5 and 21 µg (for tissue volumes from 0.07 to 1.6 mm3. Overall, a 1 x 1 mm core (0.8 mm3) was the smallest with consistent yields above the 4 µg threshold needed for replicate runs in our workflow. Cores of this dimension were prepared from 328 patient samples from 139 FFPE blocks of an oropharyngeal squamous cell carcinoma cohort, and successfully permitted duplicate runs of the whole cohort.
This study makes viable the use of TMAs or the smallest possible cores for proteomics, and defines the lower limits of FFPE sample sizes that can be reliably prepared for MS analysis. The workflow offers a new path for small samples when there is a limited tumour sample source.

P12.C31

Automated Solid-Phase Extraction Methods for High-Throughput Proteomic Sample Preparation

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Introduction: Co-eluting matrix components in clinical samples can adversely affect peptide quantitation and LC-MS spectral quality. Prior to MS analysis, solid-phase extraction (SPE) clean-up is typically required for tryptic peptide digests. This is the most labour-intensive step of sample preparation. Our aim was to develop automated SPE workflows whilst maintaining LC-MS data reproducibility.
Methods: We developed two SPE workflows using a Beckman Coulter NXP robotic workstation. Both methods automated the conditioning of the SPE plate, sample transfer, washing, and peptide elution. The first 90-minute method utilises a Waters Oasis PRiME HLB 96-well plate and is suitable for various sample sources, including fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissues. A second 120-minute workflow utilising a Waters Oasis MCX 96-well plate was needed for samples embedded in optimal cutting temperature compound (OCT) to remove polymers.
Results: The HLB method was developed using human HEK293 cell line lysates. Quantitation of peptide recovery demonstrated precision for low (25 µg, CV 11%, n = 15) and high peptide loads (130 µg, CV 3%, n = 8). This method was validated using 45 tumour and matched normal FFPE cancer tissues. The MCX method was developed using OCT embedded rat liver tissues. Quantitation of peptide recovery demonstrated good precision (110 µg, CV 13%, n = 16). This method was validated using 60 OCT sections from various human tissue types. To compare the robotic workflows with manual approaches, 12 HEK293 cell lysates were processed through either the HLB or MCX workflows across multiple sample preparation runs. The samples processed through either method on the robot returned significantly higher peptide yields than samples manually cleaned on a vacuum manifold by experienced operators.
Conclusions: Implementation of an automated workstation for proteomic sample preparation is reproducible and will play an important role in achieving the high-throughput necessary for clinical applications.

P12.C32

Targeted Analysis of Protein Biomarkers in Biological Fluids by on-Line Aptamer-Affinity Solid-Phase Extraction Capillary Electrophoresis-Mass Spectrometry

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Introduction: Enzyme-linked immunosorbent assay (ELISA), and other biosensors based on immuno-affinity, or more recently aptamer-affinity, have been widely developed and applied in the analysis of protein biomarkers for research and diagnostics. However, despite the excellent selectivity provided by the affinity ligand, these methods can be prone to false positive because of non-specific adsorption, cross-reactivity and lack of a reliable target analyte identification.

Methods: As an alternative to these methods, we propose on-line immuno- and aptamer-affinity solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS). This simple and powerful three-dimensional tool combines the high extraction selectivity of antibodies and aptamers with the high-performance separation features of CE and the uniqueness of MS detection, which allows a reliable identification of the preconcentrated and separated protein biomarkers.

Results: Here we present as a proof-of-concept a fully integrated and valve-free aptamer-affinity SPE-CE-MS method for the sensitive analysis of intact α-synuclein, which is a major component of Lewy bodies, the characteristic protein aggregates of Parkinson's disease. Under the optimized conditions with a recombinant α-synuclein standard, the method figures of merit were remarkable and the limit of detection was decreased 100 times compared to CE-MS. This excellent performance is due to the high affinity of the aptamer to the target protein and, in comparison to antibodies, to the improved aptamer tolerance to the acidic and basic conditions used for the separation and the elution.
In red blood cells lysates, N-acetylated α-synuclein, which is the most abundant proteoform in blood, was the only proteoform detected. Despite non-specific adsorption in the sorbent of mainly ubiquitin, the electrophoretic separation and reliable MS identification prevented the possibility of a false positive or an inaccurate quantification of the target protein.

Conclusions: The results point to aptamer-affinity SPE-CE-MS as a simple, selective, sensitive and accurate tool for the high-throughput targeted analysis of protein biomarkers.

P12.C34

Using of SILAC Technique for Studying Therapy-Induced Cell Communication in Ovarian Cancer Cells

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Introduction: Therapy resistance remains one of the biggest problems in cancer treatment. It has been shown that dying cancer cells emit signal molecules in the extracellular space and thus contribute to chemoresistance formation in recipient chemonaive cancer cells. We implemented a new application of the SILAC (Stable isotope labeling with amino acids in cell culture) method for secretome generation from donor cancer cells and its incubation with recipient cells.
Methods: Therapy-induced (TIS) or control secretomes (CS) were obtained from donor ovarian cancer cells with complete incorporation of a Heavy label (L-Arginine-HCl 13C6 15N4; L-Lysine-2HCl 13C6 15N2). Next, these secretomes were concentrated and resuspended in SILAC medium containing Medium amino acids (L-Arginine-HCL 13C6; L-Lysine-2HCl 4,4,5,5-D). Unlabeled cancer cells were incubated for 24 hours with TIS or CS and then were subjected to proteome analysis. An MTT assay was performed to test the chemosensitivity of recipient cells.
Results: We showed that TIS decreases the sensitivity of recipient cells to the cisplatin. Next, our LC-MS/MS analysis of cell lysates revealed a total of 4224 proteins.
An analysis of heavy-labeled proteins in recipient cells showed direct transport of a pool of proteins involved in translation, splicing, stress granules formation, and oxidative phosphorylation from dying donor cells to recipient cells. An analysis of medium-labeled proteins showed that TIS provokes an increased abundance of proteins involved in the cell cycle, RNA processing, oxidative phosphorylation, and cytoskeleton structuring in recipient cells.
Conclusion: Therapy-induced secretion of dying cancer cells leads to chemoresistance formation in recipient cells by the export and uptake of proteins that regulate translation, splicing, and metabolism. This signaling can be mediated by the stress granules as a part of communication by the means of extracellular vesicles.
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P12.C36

Glycoprotein Characterization Through Sensitive Analysis Of Glycopeptides By On-line Solid-phase Extraction Capillary Electrophoresis-Mass Spectrometry

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1.- Introduction
On-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) using titanium dioxide (TiO_2) and phenylboronic acid (PBA) sorbents was evaluated for the selective purification and preconcentration of glycopeptides from enzymatic digests of glycoproteins analysed in bottom-up proteomic approaches.

2.- Methods
Recombinant human erythropoietin biopharmaceuticals (rhEPO and neuroEPO plus) were subjected to enzymatic digestion with several proteases (trypsin, chymotrypsin and Glu-C). A particle-packed microcartridge was integrated in-line near the entrance of the CE-MS separation capillary and no valves were necessary for the operation. The sorbent was conditioned, the sample was loaded (≃100 µL) and after several washing steps, retained glycopeptides were eluted and glycopeptide glycoforms were separated and detected by CE-MS.

3.- Results
The tryptic O_{126} and N_{83} glycopeptides from rhEPO were used as reference glycopeptides to optimize both TiO_2- and PBA-SPE-CE-MS methodologies. Several aspects that affect the selective retention and elution, peak efficiency and electrophoretic separation of the glycopeptide glycoforms were investigated to maximize detection sensitivity while minimizing non-specific retention of peptides. Both SPE-CE-MS methods were validated in terms of repeatability, linearity, limits of detection and microcartridge lifetime. In addition, selectivity of both sorbents towards sialylated and branched glycoforms was also studied. Both methods presented adequate repeatability and linearity, but PBA-SPE-CE-MS showed improved preconcentration factors (up to 500-fold) and microcartridge lifetime. Finally, the PBA-SPE-CE-MS method was applied to the analysis of glycopeptides from rhEPO and neuroEPO plus biopharmaceuticals, demonstrating that the enhanced sensitivity enables an improved characterization of the glycan composition of their glycosites.

4.- Conclusions
The established SPE-CE-MS methods made possible to substantially enhance detection sensitivity of glycopeptides compared to conventional CE-MS, without compromising separation between glycoforms. Its application to study other glycoproteins that are deemed as relevant biopharmaceuticals or biomarkers for a wide variety of diseases could be also possible.

P12.C38

Quantitative Proteomics Identifies Redox Switches That Regulate Fetal and Adult Hematopoiesis

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Introduction: Fetal and adult hematopoietic stem and progenitor cells (HSPCs) are characterized by distinct redox homeostasis that may influence their differential cellular behavior in normal and malignant hematopoiesis. Despite the known function of redox signaling in controlling several cellular processes, the role of redox signaling in altering the proteotype and phenotype of HSPCs during development remains elusive.

Methods: In this study, we have applied a sequential iodoTMT labeling strategy and a nanoLC-MS3 method to characterize the redox state of cysteines in 400’000 fluorescence-activated cell sorting (FACS)-purified primary mouse fetal and adult HSPCs.

Results: We defined the redox state of 4455 cysteines in 1909 unique proteins in fetal and adult HSPCs. In agreement with the divergent nature of fetal and adult hematopoiesis, we show that the redox molecular landscape is distinct between fetal and adult HSPCs, and forms an additional layer of regulation of HSPCs along ontogeny. We demonstrate that cysteine proteins in fetal HSPCs are more prone to redox modulation than in adult HSPCs. Our data identified ontogenically active redox switches in proteins with a pronounced role in proliferation, metabolism and mRNA translation. Our further molecular analyses accentuate a functional impact of protein oxidation on key players of mitochondrial respiration, mRNA translation initiation as well as translation re-initiation, and suggest their involvement during development and in leukemia.

Conclusions: Our data show that the pro-oxidative environment in fetal HSPCs is an ontogeny-specific feature crucial for the regulation of developmental processes by redox signaling, while at the same time, it makes fetal HSPCs more vulnerable to increased exposure to ROS. This work significantly contributes to further understanding of redox signaling in developmental and malignant hematopoiesis.

P12.C39

Development of Immunoaffinity-Selected Reaction Monitoring Assays for the Differential Quantification of Human Endogenous Retrovirus Proteins

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Introduction
Highly homologous human endogenous retrovirus (HERV) sequences constitute nearly 8% of the human genome, but only a limited number of studies have assessed the expression of HERVs proteins and their functional role. Envelope proteins of HERVs group K (HERV-K env) are cell membrane proteins which could be shed into biological fluids and emerge as promising disease biomarkers. However, due to the high homology and potential cross-reactivity, differential quantification of HERV-K env proteins and their evaluation as biomarkers can hardly be achieved by immunoassays. To address this limitation, we proceeded with the development of sensitive and highly specific immunoaffinity-mass spectrometry (IA-SRM) assays for the differential quantification of HERV-K env proteins.
Methods
RT-PCR was performed using primers from previous literature to assess the expression pattern of HERV-K env genes at the transcript level. Two commercial antibodies (HERM 1811-5 and ERVK-7) targeting highly conserved regions were used to capture 13 different HERV-K env proteins. Following IA enrichment, shotgun bottom-up proteomics using high-resolution nanoLC-MS/MS was performed. Following this, 24 stable-isotope labeled peptide internal standards were designed to enable differential quantification of HERV-K env proteins by nanoLC-SRM.
Results
The 24 internal standard peptides were optimized by adjusting SRM transitions, collision energy, charge, and LC gradient to enable accurate detection of HERV-K env proteins. Expression of HERV-K proteins was evaluated in four different cell lines (MCF-7, MDA-MB-231, LNCaP, and H9 cells).
Conclusions
IA-MS assays could emerge as exclusive tools for investigation of the “dark matter” of the human proteome, which could not previously be achieved with either immunoassays or mass spectrometry on their own due to the high homology of such proteins. Our assays may enable us to evaluate whether HERV-K env genes are expressed at the protein level and next to assess if they can be a promising class of disease biomarkers.

P12.C40

Development of a Peptidome Analysis Method for Submilligram Brain Tissue

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Introduction: The Living organisms contain a variety of endogenous peptides that function as significant regulators of many biological processes. However, LC-MS–based peptidomics studies have not facilitated an understanding of the individual differences and tissue specificity of peptide abundance because the low efficiency of peptide extraction and low abundance of peptides in a single animal. In this study, we established a method to analyze peptides in detail from a small amount of tissues.
Methods: A mouse brain was frozen immediately after dissection and then sliced. Peptides were extracted from a frozen slice of hypothalamus using the modified differential solubilization (DS) method (1, 2). Peptide extracts derived from an equivalent of 135 µg of hypothalamus was analyzed using Q-Exactive equipped with an EASY-nLC 1000 system (Thermo Fisher Scientific). LC-MS/MS data were searched against the mouse UniProt database (reviewed, canonical; 17,053 entries, release 2020_3) using PEAKS X Studio (Bioinformatics Solutions Inc.).
Results: LC-MS/MS analysis resulted in the identification of 1,535 peptides derived from 297 proteins. Approximately 45% of the identified peptides (690 of 1,535 peptides) belonged to prohormone precursor protein groups. Within the prohormone precursor protein group, 35 bioactive peptides listed in the database, including substance P, neurokinin A, neuropeptide K, neuropeptide Y, lipotropin γ and α-MSH, were identified. Furthermore, as for the neuropeptides secreted from the hypothalamus, Orexin B, β-endorphin and three opioids α-neoendorphin, dynorphin A and dynorphin B were detected.
Conclusions: The method described here for small samples of tissue from a single animal may facilitate the discovery of novel bioactive peptides and disease related peptides via highly reproducible quantitative analyses.

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P12.C42

Comprehensive Proteomic Characterization of the Intra- And Extracellular Adaptations in Response to Oxidative Stress by OxSWATH

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Introduction: In this work, we applied our novel approach, the oxSWATH[1], to perform an exhaustive characterization of the intracellular and extracellular proteomic alterations of cells exposed to oxidative cells. This method allowed the integration of the data regarding relative cysteine oxidation with the analysis of the total protein level. Thus, in a single analysis, it was possible to evaluate the alteration considering the redox status of the proteins and perform a generic differential protein expression analysis.
Methods: To completely characterize the cellular response to acute stimulation with hydrogen peroxide, the cellular proteome and the secretome were analyzed using the oxSWATH method, covering the intra- and extracellular responses, respectively.
Results: A total of 915 proteins were altered upon oxidative stress, from which 90 were altered in both intra- and extracellular space. Moreover, a clear tendency for remodeling the extracellular space was observed, with nearly 80% of the altered proteins found altered in the secretome. The analysis of the overall redox status of the proteins revealed a tendency to have a reduced environment in the extracellular space, while an equilibrium between the reduced and oxidized proteins is achieved in the intracellular environment. Again, a higher number of secreted proteins alter their redox status upon oxidative stress compared with the intracellular protein (250 and 61 proteins, respectively). From those, only 4 proteins were commonly altered between the two cellular spaces.
Conclusions: Overall, these results point for a differential adaptation of the intracellular and extracellular proteomes, with the extracellular space being particularly affected by oxidative stress. Moreover, the potential of the oxSWATH method was proved since a truly comprehensive evaluation of proteomics changes upon the oxidative stimulus was achieved using a single approach.

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P12.C44

Automated, Parallel Protein Extraction for Analysis of Low Input FFPE, Fresh Tissue and Cells Clinical Samples with Adaptive Focused Acoustics

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Title: Standardized Sample Preparation Workflows for Clinical Proteomics
Introduction: Many research projects in translational or clinical laboratories require automated, hands-off solutions for protein sample preparation which enable better reproducibility, increased efficiency, higher quality results, and faster turnaround time. This poster introduces Adaptive Focused Acoustics (AFA) for single-pot, simultaneous multi sample processing from diverse inputs in various format for formalin-fixed and paraffin-embedded (FFPE) tissue, Laser Capture Microdissection (LCM), fresh frozen tissue, and cultured cells for mass spectrometry-based (MS) proteomics.
Methods: Different sample types (Mouse liver, Pig heart, Mouse Kidney…) were processed for protein extraction with AFA. The strips and plates formats allow for easy dispensing of difficult inputs like LCM and streamlined processing through a single pot handling. The extraction process is fully compatible with a single pot approach, e.g. using SP3 (single pot solid phase sample preparation) to clean up and digest the proteins.
Results: The employed protein extraction and analysis workflow displays highly consistent and reproducible results for the various sample inputs tested (laser captured microdissections, cultured cells, fresh tissue and FFPE samples). Some critical steps like deparaffinization can be handled without the need of toxic solvents, and in a much faster way. CVs are limited to below 15% (of which half can be attributed to the MS analysis part) and Pearson correlations are above 0.95, for all sample types.

Concusion: AFA-assisted sample preparation is a fast, robust approach for processing hundreds of samples within a week, enabling reproducible studies in pre-clinical and clinical research, making it ideal for:
• Samples from the clinic, such as fresh frozen tissue material,
• Samples from pathology or biobanks such as PFA, FFPE, LCM or DBS samples,
• Targeted assays for marker protein identification.

P12.C45

HTPS: A Proteomic High-Throughput Screen to Map Specificity, Cleavage Entropy, Allosteric Changes and Substrates of Proteases

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Introduction:
Proteases are among the largest protein families and critical regulators of biochemical processes like apoptosis and blood coagulation. Knowledge of proteases has been expanded by the development of proteomic approaches, however, technology for multiplexed screening of proteases within native environments is currently lacking behind.
Methods:
We introduce a proteomic workflow (HTPS) to profile protease activity based on isolation of protease products from native lysates using a 96FASP filter, their identification in a mass spectrometer and a custom data analysis pipeline.
The method is significantly faster, cheaper, technically less demanding, easy to multiplex and produces accurate protease fingerprints.
Results:
We benchmark this method with blood cascade proteases: we obtain protease substrate profiles to map specificity, cleavage entropy allosteric changes. As well, we apply this method to investigate uncharacterize/poor studied viral proteases.
Conclusion:
The data show that protease substrate predictions enable the identification of potential relevant physiological substrates for subsequently targeted validation in biochemical assays.
References:
"Mapping specificity, cleavage entropy, allosteric changes and substrates of blood proteases in a high-throughput screen." Nat Commun. 2021 Mar 16;12(1):1693.

P12.C47

Mapping Protein Complexes for Unraveling the Hidden Proteome in Ovarian Cancer.

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Introduction: Eukaryotic mRNA is considered as monocistronic, translating a single reference protein (RefProt) from an open reading frame (ORF). Furthermore, large-scale proteomics relies on the interrogation of Databases for protein identification. However, a significant fraction of good quality spectra does not match any RefProt. OpenProt Database predicts alternative proteins (AltProt) translated from 5'-&3'-UTR, long non-coding RNAs or in frameshift. Crosslinking-mass spectrometry (XL-MS) is an attractive technique to identify networks and pathways involving AltProts. Here, this strategy was applied to decipher the roles of AltProt in the pathology of ovarian cancer.

Methods: Protein extracts from ovarian cancer cell lines (PEO-4 & SKOV-3) and inmortalized ovarian cells (SV-40) were analyzed by bottom-up to identify the abundance variation of AltProt and RefProt, using LFQ node of ProteomeDiscoverer2.5. To identify protein interactions, DSSO crosslinkers and sequential digestions (trypsin and chymotrypsin) were used in combination with nuclei enrichment. Interaction network and GO-term of AltProt and RefProt were generated via Cytoscape and ClueGo.

Results: 7,512 RefProt and 453 AltProt were identified by combining two complementary extraction methods (SDS 1% and RIPA). Using principal component analysis, the samples for each cell line in this study clustered together, for AltProts, which was consistent with results from RefProt. Moreover, we found a significant variation and specific assemblages of AltProt and RefProt abundance between cancer and immortalized cells. The crosslinking network highlighted several networks involving AltProts.

Conclusions: Our AltProt abundance variation analysis highlights the involvement of these proteins in ovarian cancer. Deciphering the precise function of AltProts can be inferred from a crosslinking network, followed by String and GO-term enrichment. Further improvements on the crosslinking technique will be crucial to obtain more robust networks. Moreover, validation of the identified interactions by orthogonal methods (BioID & Virotrap) will be important as well.

P12.C48

Developing a Targeted Mass Spectrometry Workflow for Investigating the Tear Proteome from Healthy Volunteers

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Introduction
Proteins in tears play an important role in eye health. Previous work has shown proteins to be a promising source of biomarkers involved in different eye pathologies. The goal of this study was to develop a robust and sensitive method for profiling tear proteins on healthy volunteers, to examine the variability in a healthy population. The application of this method will be used to help diagnose and stage certain eye diseases

Method
Tear samples were collected on tear strips followed by tryptic digestion for analysis using a targeted method of 596 proteins with scheduled multiple reaction monitoring (LC-sMRM) on a Sciex QTRAP 5500 platform. These targeted proteins were compiled from high-resolution MS/MS data previously acquired in data-dependent (IDA) and data-independent mode (SWATH) on a Sciex TripleTOF 5600+ platform.

Results
LC-MRM method was develop based on our in-house database of 613 proteins groups identify from IDA analysis. Raw data were processed using ProteinPilot 5.0 (Sciex) to identify proteins at 1% FDR. Optimal MRM transitions were selected for each peptide of interest. A final list of 596 proteins were targeted with two sMRM methods. 226 proteins showed consistent peak shape and signal-to-noise and were chosen to investigate interindividual variations in 16 healthy volunteers as well as intra-day variability in 9 healthy volunteers.

Conclusion
This study will help to better ascertain the normal variation of proteins in tears for future work to find potential biomarkers of eye diseases.

P12.C49

Phosphoproteomic Workflow Optimization for the Analysis of FFPE Tissue Sections

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Introduction: For efficient investigation of phosphorylation, it is inevitable to perform enrichment and purification steps before analysis to increase sensitivity towards phosphopeptides. Our goal was to develop and optimize these sample preparation methods to apply them effectively to small-size FFPE tissue samples.
Methods: Commercial HeLa cell line tryptic digest was used for method development. Phosphopeptide (PP) enrichment of a small amount of starting material was developed testing Pierce TiO2 pipet tips and a TiO2 coated monolithic column against several loading buffers (eg. lactic acid, TFA, citric acid). For PP-optimized sample purification, Pierce C18 spin column was used following minor optimization in the loading and elution conditions. For exploring the dynamic range of the developed enrichment method, rat smooth muscle digest was used.
On-surface tryptic digestion, C18 clean-up, and PP enrichment were performed on formalin-fixed paraffin-embedded (FFPE) lung tissue sections. After RP-HPLC-MS/MS measurements, Byonic, GlycoPattern, and SkyLine software were used for data analysis.
Results: Among all the tested methods, the use of pipette tip-based TiO2 stationary phase and the loading buffer containing 50 mM citric acid/1.5% TFA proved to be efficient with good repeatability. For C18 clean-up, cooled sample loading with 0.1% heptafluorobutyric acid resulted in ca. 30% recovery increase compared to the manufacturer's protocol. After the method development with 500 ng complex mixtures, we examined method performance in a wider (1-40 µg) range, obtaining excellent results. Finally, the developed methodology was applied to FFPE tissue samples. Between adenocarcinoma and healthy samples, several individual or differentially expressed PPs were identified.
Conclusions: We developed sample-preparation methods for enrichment of PPs from small (500 ng – 40 µg) sample size that are applicable for the examination of phosphorylation in small lung tissue sections.
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P12.C50

Comparison of In-Solution and S-TrapTM Based Sample Preparation for Tear Proteomics Study

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Introduction
Sample preparation methods for mass spectrometry vary significantly. Suspension trapping (S-TrapTM) method was reported as an effective way of proteomic sample preparation in various sample types. Yet, this new approach has not been well-studied on human tear fluids. This study aims to compare the workflow and tear proteome identified using conventional in-solution approach and this spin-column based approach.

Methods
About 100 µl of tears from five healthy adults (n=10 eyes) was collected using disposable Strip Meniscometry Tube (SMTube®). After protein assay. equal amount of samples were pooled to form a grouped lysate. Equal amount of sample was processed for protein extraction, reduction, alkylation and digestion using either conventional in-solution protocol or S-trapTM protocol in parallel. Six technical replicates were performed for each preparation to assess the protein identification reproducibility using a Triple TOF ® 6600 mass spectrometer (SCIEX). Proteins was identified at 1%FDR using ProteinPilot 5.0 (SCIEX) with Gene ontology (GO) compared using PANTHER classification system.

Results
The S-Trap protocol requires less preparation time than a typical in-solution digestion. Both methods showed good reproducibility within technical replicates, with the peptide recovery yield significantly higher in S-trap group than that of in-solution group (74.24±4.95% vs 52.8±1.58%).
The IDA search identified 1757 and 1267 proteins in S-trap group and in-solution group respectively. Among them, 798 proteins were commonly found in both protocols. Yet, GO analysis revealed very similar proteomes from the two approaches, in which binding, catalytic activity and molecular function regulator were their main molecular functions.

Conclusions
S-TrapTM protocol outperformed in-solution protocol in terms of preparation time, protein recovery and total protein identification in this study. The workflow established can be applied in tear biomarker research for studying ocular diseases.

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P12.C51

DeGlyPHER: An Ultrasensitive Method for Analysis of Viral Spike N-Glycoforms

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Introduction:
Viruses can evade the host immune system by displaying numerous glycans on their surface "spike-proteins" that cover immune epitopes. We have developed an ultrasensitive "single pot" method to assess glycan occupancy and the extent of glycan processing from high-mannose to complex forms at each N-glycosylation site. Though aimed at characterizing glycosylation of viral spike-proteins as potential vaccines, this method is applicable for analysis of site-specific glycosylation of any glycoprotein.

Methods:
In a "single pot", using a single protease, and sequential treatment with endoglycosidases that create residual mass signatures identifiable by ESI-LC-MS/MS, performed in suitable volatile buffers, we broadly characterize the nature of the N-glycosylation, determining the degree of glycan occupancy, and the degree of glycan processing - the initially attached high mannose form, and which may mature into the complex form when mannose residues are replaced by "terminal" monosaccharide sequences.

Results:
Using progressively decreasing amounts of starting material, ranging from 1 microgram to 5 nanograms, we observed that a single ESI-LC-MS/MS run with 1 microgram of starting material was enough to cover >95% of the amino acid sequence and all N-glycosylation sites, which is 90 times more sensitive than our previous approach. DeGlyPHER is agnostic to mass spectrometry platform - a timsTOF Pro mass-spectrometer coupled to an Evosep One HPLC was used to achieve >99% sequence coverage and identification of all N-glycosylation sites using a single ESI-LC-MS/MS run with 0.5 microgram of starting material and an 88-minute LC gradient. Thus, the sensitivity of DeGlyPHER on this platform was 180 times higher than our previous approach.

Conclusion:
Our strategy is much more sensitive, rapid, and simple (sample processing and computation) than existing "intact glycopeptide" analytical strategies that have been used for such analyses, with up to 180-fold increase in sensitivity, while maintaining >95% sequence coverage and identifying all N-glycosylation sites.

P13.02

Identifying Disease-Induced Interactome Changes in the Honey Bee Midgut

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Introduction
Honey bees are important insect pollinators, but their health is threatened by several understudied pathogens. The microsporidian parasite Nosema ceranae is one of the prevalent pathogens that poses a threat to bee health. Nosema spores rapidly proliferate in bees' epithelial midgut cells, weakening their immune systems by depleting their nutrients and subsequently spreading throughout the colony, causing disease. The resulting symptoms of the diseased colony have been attributed to colony death. My thesis aims to elucidate Nosema's mechanism of action in the honey bee midgut. The work I describe here probes the protein interaction network of the honey bee midgut to observe changes induced by Nosema infection.

Methods
We have used a co-elution strategy called protein correlation profiling (PCP), a powerful proteomics method that combines size exclusion chromatography (SEC) with mass spectrometry to isolate and identify possible protein complexes. Well established coelution strategies have been successfully used in our lab to map the interactome of mammalian cells and organisms. The honey bee on the other hand, is a non-model organism whose interactome had previously been uncharacterized. We utilized PCP to remedy this problem and obtained a first snapshot of the protein-protein interaction network of the honey bee midgut.
Using this information, we had a basis upon which we could explore Nosema's infection dynamics on the bee midgut interactome.

Results
In our preliminary findings, we identified protein correlation profiles that are common between control and treatment conditions, as well as profiles that are different between the two conditions, indicating that the observed changes between the compared protein interaction networks is due to Nosema infection. We will validate these findings using complementary techniques.

Conclusion
This work highlights novel results obtained from observing changing protein-protein interactions in honey bee midguts upon infection with Nosema.

P13.03

Molecular Weight-Based Proteome Fractionation by Stepwise Organic Solvent Precipitation

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Introduction: The Cohn process is a classic approach to fractionate plasma proteins by stepwise adjustments to the pH and ethanol solvent content of the sample. While organic solvent precipitation remains a favorable approach to concentrate and purify proteome samples, precipitation is no longer employed to fractionate proteins, as researchers have transitioned to other approaches such as chromatography. Our group recently reported on a rapid acetone precipitation protocol that exploits higher temperature and ionic strength for quantitative recovery of proteome samples in minutes [1]. We later demonstrated the optimal recovery of low molecular weight proteins and peptides by elevating the organic solvent content in combination with zinc sulfate [2].

Methods: A whole proteome extract from S. cerevisiae was selected as a model system. Proteins were precipitated in a variety of conditions ranging in organic solvent content, organic solvent type, salt type and time courses. Molecular weight profiling of the recovered fractions was visualized by SDS-PAGE, and the recovery of precipitated proteins was assessed with LC-UV. Also, fractionated proteins were subjected to bottom-up MS analysis, to characterize the resulting mixture and confirm protein properties that correlate to their precipitation efficiency.

Results: The results indicate different protein precipitation efficiency by variations in acetone content and salt type. High molecular weight proteins precipitate more readily than low molecular weight proteins in lower acetone concentrations. Moreover, lower molecular weight proteins precipitate by increasing acetone concentration and using zinc sulfate salt. Based on these results, a stepwise precipitation protocol was optimized to separate proteins as a function of molecular weight.

Conclusion: The proposed protocol has shown the potential of precipitation as a rapid fractionation technique to separate proteins as a function of molecular weight ahead of MS analysis.

1- Nickerson, J. Proteome Res, 2020, p2035.
2- Baghalabadi, Anal Chim Acta, 2020, v1138 p38.

P13.04

Conservation and Conditional Regulation of Protein Ubiquitination

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Introduction: Protein ubiquitination is a complex modification, canonically linked to proteasome mediated degradation but also related with other cellular processes. In the last decade non-degradative ubiquitination have been exemplified but remain difficult to study. Here we present a systematic study of the relation between site conservation and non-degradative functions.
Methods: We compiled published data containing ubiquitination sites from 27 MS-based studies, from 8 different species and quantified upon different stimuli in Homo sapiens. Highly conserved sites were defined as those conserved within regions of the same protein across species or enriched in the same positions of members of protein families. We selected 16 conserved sites not affected by proteasome inhibition that were mutated in yeast to arginine and tested for growth phenotypes in 41 different conditions .
Results: Our dataset contains ~160,500 ubiquitination sites (~110,000 in human, ~62,000 quantified) from ~26,500 different protein sequences (~11,000 in human). Ubiquitination site quantitation under different stimuli showed high correlation among different proteasome and DUBs inhibitors and anticorrelation with other stimuli such as DNA damage. Highly conserved ubiquitination sites tend to be less affected by proteasome inhibitor treatment and more by other stimuli such as DNA damage suggesting that non-degradative ubiquitination tends to be more conserved. We narrowed down a list of sites that were both highly conserved and not affected by proteasome inhibition which we hypothesise will be enriched in non-degradative ubiquitination. We then measured growth phenotypes in yeast mutants at these positions finding several candidates with significant phenotypes, suggesting non-degradative functions.
Conclusions: sites that are highly conserved are less affected by proteasomal degradation and represents a potential pool of non degradative regulatory sites. K to R mutation in yeast of some of these positions resulted in significant fitness changes compatible with our hypothesis.

P13.05

Proteome Analysis Reveals Pathways of Corticoid- And Shape Constraint-Induced Transdifferentiation of HepaRG Cells

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Introduction:
HepaRG cells are the most appropriate and versatile cell system surrogate for primary human hepatocytes [1]. They exhibit unique properties: self-renewal of progenitor cells, full differentiation toward hepatocytes or cholangiocytes, and an ability for retro-differentiation toward a proliferative state. To unravel the molecular mechanisms controlling such plasticity and HepaRG transdifferentiation abilities, the proteomes of several cell lines derived from HepaRG progenitors were compared.

Methods:
From HepaRG progenitors, stem-like cells (HepaSC) were produced using shape constraint, and differentiated hepatoblasts were obtained from HepaSC cells via a reference hormonal pathway (HepaED) or via shape constraint and a corticoid treatment (HepaRP). Cell lines were subjected to quantitative label-free proteomics using a TIMS-TOF Pro coupled to a NanoElute (BRUKER). Identifications and quantifications were performed using Maxquant software. ANOVA and Tukey's HSD tests were used to assess statistically significant differences in protein abundance between cell lines.

Results:
From the robust identification of 5703 proteins, intensity-based label-free quantification was performed for the 3449 of them that fulfilled stringent validation criteria. Statistical analysis highlighted 1407 differentially-expressed proteins, and functional annotation analysis allowed showing main changes in HepaRP cells for structural proteins known to be in involved in mechanosensing, but also for markers of the differentiation state. Unlike mechanotransduction due to shape constraint, the TGF-β cascade was confirmed as key pathway for the hormonally-induced differentiation of HepaRG cells. Cell lines also exhibited differences in terms of the abundance of stress-related and chromatin-remodeling markers.

Conclusions:
Proteomics was able to discriminate between the differential differentiation pathways in HepaRG cells induced by a reference hormonal treatment or by mechanotransduction and a corticoid treatment. Differences also highlight how mechanotransduction may favour a higher genome stability, thus increasing the sustainability and reproducibility of our hepatocyte-like model cell system.

[1] Tascher et al. Cells 2019, 8, 192; doi:10.3390/cells8020192

P13.06

Cancer Stem Cell Marker DCLK1 Reprograms Small Extracellular Vesicles toward Migratory Phenotype in Gastric Cancer Cells

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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 (GC). DCLK1 overexpression in a majority of solid cancers correlates with lymph node metastases, advanced disease and overall poor-prognosis. In cancer cells, DCLK1 expression has been shown to promote epithelial-to-mesenchymal transition (EMT), driving disruption of cell-cell adhesion, cell migration and invasion. Here, we report that DCLK1 influences small extracellular vesicle (sEV/exosome) biogenesis in a kinase-dependent manner in regards to sEV size and amount secreted. In addition, sEVs isolated from DCLK1 overexpressing human GC cell line MKN1 (MKN1oe-sEVs), promote the migration of parental (non-transfected) MKN1 cells (MKN1par). Quantitative proteome analysis of MKN1oe-sEVs revealed enrichment in migratory and adhesion regulators (STRAP, CORO1B, BCAM, COL3A, CCN1) in comparison to MKN1par-sEVs. Moreover, using DCLK1-IN-1, a specific small molecule inhibitor of DCLK1, we reversed the increase in sEV size and concentration in contrast to other EV subtypes, as well as kinase-dependent cargo selection of proteins involved in EV biogenesis (KTN1, CHMP1A, MYO1G) and migration and adhesion processes (STRAP, CCN1). Our findings highlight a specific role of DCLK1-kinase dependent cargo selection for sEVs and shed new light on its role as a regulator of signaling in gastric tumorigenesis.

P13.07

Quantitative Phosphoproteomics Reveals Ectopic ATP Synthase on Mesenchymal Stem Cells to Promote Tumor Progression via ERK/c-Fos Pathway Activation

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Introduction: The tumor microenvironment (TME), which comprises cellular and noncellular components, is involved in the complex process of cancer development. Emerging evidence suggests that mesenchymal stem cells (MSCs), one of the vital regulators of the TME, foster tumor progression through paracrine secretion. However, the comprehensive phospho-signaling pathways that are mediated by MSCs-secreting factors have not yet been fully established.
Methods: To dissect the MSCs-triggered phosphorylated network, we applied quantitative phosphoproteomics using lung cancer cells treated with MSCs-conditioned medium (MSC-CM), and analyzed the proteins with differentially phosphorylated status to determine MSCs-activated pathways.
Results: In phosphoproteomic profiling, a total of 1995 phosphorylation sites are identified in lung cancer cells stimulated with MSC-CM. Integrative analysis of the identified phosphoproteins and predicted kinases demonstrates that MSC-CM functionally promotes the proliferation and migration of lung cancer via the ERK/phospho-c-Fos-S374 pathway. Recent studies have reported that extracellular ATP accumulates in the tumor microenvironment and stimulates the P2X7 receptor on the cancer cell membrane via purinergic signaling. We observe that ectopic ATP synthase is located on the surface of MSCs and excreted extracellular ATP into the lung cancer microenvironment to trigger the ERK/phospho-c-Fos-S374 pathway, which is consistent with these previous findings.
Conclusions: Our results suggest that ectopic ATP synthase on the surface of MSCs releases extracellular ATP into the tumor microenvironment, which promotes cancer progression via activation of the ERK/phospho-c-Fos-S374 pathway.

P13.09

NanoLC-nESI/MS/MS Analysis of Malondialdehyde-Induced Post-Translational Modifications in Breast Cancer Patients

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Introduction: Malondialdehyde (MDA) is a reactive aldehyde generated from endogenous peroxidation of polyunsaturated fatty acids. Malondialdehyde-induced damages of cellular DNA and proteins are associated with several pathological conditions and diseases. Breast cancer patients are known with elevated lipid peroxidation/oxidative stress. Post-translational modifications of blood hemoglobin have been used as biomarker of exposure to chemicals.
Methods: Two types of malondialdehyde-induced modification, namely the Schiff base and the dihydropyridine (DHP), were identified at various sites in the peptide digest of human hemoglobin by the high-resolution mass spectrometry. The relative extents of the dose-responsive modifications were simultaneously quantified in globin isolated from the blood of breast cancer patients and the control subjects by the nanoflow liquid chromatography nanoelectrosrpay ionization tandem mass spectrometry under selected reaction monitoring (nanoLC-nESI-MS/MS-SRM).
Results: Totally. 14 Schiff base- and 9 DHP- types of MDA-induced modifications in human hemoglobin were identified. The Schiff base and the DHP types of modification led to the mass increase of 54 and 134 amu, respectively, at mainly the lysine and histidine residues. The degrees of modification increase dose-dependently in ten of the Schiff base- and four of the DHP-sites. Thus, the relative extents of these 14 dose-responsive modifications were simultaneously quantified by nanoLC-nESI-MS/MS-SRM. The results indicated that DHP formation at β-Lys-59 is significantly higher in hemoglobin isolated from the blood of breast cancer patients than that in healthy female subjects ($p < 0.05$).
Conclusions: Starting from one drop of blood, measuring DHP formation at β-Lys-59 in hemoglobin might represent useful biomarkers for MDA-induced protein damage in breast cancer.

P13.10

A Virus-Host Protein Interactome Comparison of Differentially Pathogenic Arenaviruses

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Introduction: The pathogenicity of zoonotic viruses differs greatly even for closely related virus species of the same genus. We hypothesize that these differences in pathogenicity are at least in part determined by differences in the host interactomes of viral protein homologs from these pathogenic and non-pathogenic virus species. In order to study this, we used immunoprecipitation mass spectrometry (IP-MS) as an open view method to investigate major differences and similarities in the viral protein interactomes between the pathogenic arenavirus Junín virus (JUNV) and the closely related but non-pathogenic Tacaribe virus (TCRV).
Method: Human embryonic kidney cells were transfected with plasmids encoding FLAG-tagged recombinant versions of the viral matrix protein (Z), nucleoprotein (NP) or glycoprotein (GP) from either JUNV or TCRV, respectively. Pull-downs of the viral proteins were on-bead digested and subsequently measured with high resolution mass spectrometry in combination with label-free quantitation. In order to find high confidence interacting host protein hits, the Mass Spectrometry interaction STatistics (MiST) score was calculated. Qualitative and quantitative data were used to identify candidates for follow-up research.
Results: Resulting protein lists are mapped to the corresponding genes and evaluated by Gene Ontology (GO) term enrichment analysis. An interactome map displays several high confidence interacting protein candidates based on the MiST score for the viral proteins of both TCRV and JUNV.
Conclusion: Homologous viral proteins share several protein interaction candidates or similar protein categories, as well as enriched GO terms. Furthermore, the interactome map provides host protein candidates for follow-up research based on qualitative and quantitative differences between the pathogenic JUNV and non-pathogenic TCRV.

P13.11

New Proteomics Insights in the Characterization of FACs-Sorted Leukocyte-Derived Extracellular Vesicles as "Liquid Biopsy" of Immune Response

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Introduction: Extracellular Vesicles (EVs) are nano-vesicles released by various cell types. Proteomics approaches are emerging as promising tools for EVs protein cargo characterization. However, their isolation from whole biofluids is extremely difficult for many reasons especially linked to the presence of circulating abundant proteins that may influence the purity of EVs impairing the quality of proteomics results. We recently optimized an innovative protocol for the isolation and subsequent proteomics characterization of EVs from untouched biofluids by Fluorescence-Activated Cell Sorting (FACS). This method offers the great possibility of separating cellular specific EVs by sub-typing them with an appropriate panel of antibodies. Therefore, an update of our method provided a successful proof-of-concept of the proteomics characterization of FACS-sorted Leukocyte-derived EVs.
Methods: Leukocyte-derived EVs were separated by FACS (FACSAria III) staining peripheral blood (PB) and tears samples with a lipophilic cationic dye and Fluorescein-isothiocyanate (FITC)-conjugated phalloidin and CD45-Brilliant-Violet 510 (BV510)-conjugated. Biological samples were collected from Multiple Sclerosis patients and healthy controls. The EVs proteome was evaluated by nanoLC-Orbitrap-Fusion-Tribrid Mass Spectrometer. Quantitative proteomics data obtained from MaxQuant were used for functional analysis through Ingenuity Pathway Analysis (IPA).
Results: We highlighted, for the first time, that both in PB and in tears Leukocyte-EVs carry an active protein cargo able to trigger specific cellular information relating to "leukocyte mediated immunity" (FDR=4.27x10-33) according to the quantification of specific proteins involved in the recruitment and chemotaxis of leukocytes, such as protein-S100-A7, S100-A8, and S100-A9. Surprisingly, in lacrimal EVs, one of the most significant predicted Upstream Regulators was Oncostatin-M (p-value=3.51x10-11) which is involved in cytokines production and "Immune System signaling" (p-value=3.81x10-10).
Conclusions: Our proteomics data confirm that Leukocyte-derived EVs could be considered a platform for "liquid biopsy" useful in the assessment of EVs clinical significance to better understand the Immune System machinery in both physiological and pathological conditions.

P13.15

The Effects of Testosterone Replacement in a Pharmacologically Induced Hypogonadism Cohort: A Controlled Study with Healthy Young Males

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Introduction: Hypogonadism is a common health problem in men that increases with age and comorbidities, such as diabetes and obesity. Low testosterone level is associated with erectile dysfunction, decreased muscle strength, cognitive impairment, and mood disorders. Testosterone replacement therapy (TRT) has increased worldwide. However, the metabolic aspects of hypogonadism and the effect of TRT are not well understood. Therefore, the present study investigated the metabolic profile of pharmacological induced hypogonadism in healthy young males and the impact of TRT.
Method: Thirty healthy men between 19 and 32 years old were submitted to an androgen deprivation therapy (ADT) followed by TRT after 3 weeks. Blood samples were collected before ADT, 3 weeks after ADT, and two weeks after TRT. Metabolomics was performed in the plasma by liquid chromatography-high resolution mass spectrometry (LC-HRMS).
Results: In our study, a total number of 707 compounds were identified, including 368 quantified with statistical difference (q-value < 0.05, ANOVA paired). The comparison between the ADT and TRT group revealed 101 molecules with a p-value < 0.05, among them, 83 was restored by TRT. Carnitine and amino acid metabolism are the major metabolic pathways altered by testosterone levels. In this regard, we revealed that acylcarnitines, aromatic amino acids, and common markers of kidney function might be used as novel potential biomarkers induced by testosterone.
Conclusions: Hypogonadism induced by androgen deprivation therapy in healthy young males promotes several metabolic alterations partially restored by testosterone therapy. Metabolomics is a powerful technique for hormone dysfunction and TRT monitoring.

P13.16

SpatialOMx on Intracellular Bacteria Reveals Metabolic and Proteomic Phenotypes In-situ

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Introduction:
In host–microorganism associations, where the environment allows symbiotic bacteria to provide nutrition for the host, extreme bacterial genomic strain-variation can lead to an immense heterogeneity of bacterial phenotypes. Current methods don't allow for a differentiation of phenotypically different strains. SpatialOMx methods like spatial metabolomic and proteomics provide a unique potential to reveal such heterogeneous distribution of different strains and therefore phenotypes in host-microbe associations ranging from marine symbioses to the human gut.

Methods:
Frozen mussel sections of Bathymodiolus azoricus were sliced with 10 µm thickness and mounted on IntelliSlides (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Slides were coated with MALDI matrix using a TM-sprayer (HTX Technologies, Chapel Hill, USA) and measured on a timsTOF fleX MALDI-2 (Bruker Daltonics). MALDI Imaging experiments were performed on lipid/ metabolite level. A statistical segmentation showed regions of interest which were selected in SCiLS™ Lab (Bruker Daltonics) and coordinates were transferred to the LEICA LMD 7000 device and cut out. Afterwards 4D-Proteomics workflow was performed.

Results:
We detected different, spatially segregated phenotypes (chemotypes) originating from bacterial metabolism among genetically nearly identical intracellular symbionts of a marine invertebrate. Our mass spectrometry imaging results revealed two major chemotypes on the lipid level. To link the molecular machinery behind the heterogeneous metabolite production to the intracellular microbes, we further analyzed both chemotypes with spatially targeted proteomics. Applying our novel pipeline of spatial metabolomics-guided laser capture micro-dissection we detected most of the key proteins, encoded in the bacterial genomes and host proteins from those minute samples from the immediate host-microbe interface.

Conclusion:
SpatialOMx® is a powerful tool to reveal and link the metabolic pathways that drive hidden phenotypic heterogeneity that is critical for the understanding of host-microbe interactions and can ultimately discover pathogenic infections in humans as next step.

P13.19

Profiling the Human Phosphoproteome to Estimate the True Extent of Protein Phosphorylation

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Introduction

Mass spectrometry-based phosphoproteomics allows large-scale generation of phosphorylation site data. However, analytical pipelines need to be carefully optimised to minimise incorrect identification of phosphopeptide sequences or wrong localisation of phosphorylation sites within those peptides. Public databases such as PhosphoSitePlus (PSP) and PeptideAtlas (PA) compile results from published papers or openly available MS data, but to our knowledge, there is no database-level control for false discovery of sites, subsequently leading to the likely overestimation of true phosphosites. It is therefore difficult for researchers to assess which phosphosites are “real” and which are likely to be artefacts of data processing.

Methods

By profiling the human phosphoproteome, we aimed to estimate the false discovery rate (FDR) of phosphosites based on available evidence in PSP and/or PA and predict a more realistic count of true phosphosites. We ranked sites into phosphorylation likelihood sets based accumulated evidence and analysed them in terms of amino acid conservation across 100 species, sequence properties and functional annotations of associated proteins. We demonstrated significant differences between the sets and developed a method for independent phosphosite FDR estimation.

Results

We estimated a false discovery rate of 86%, 95% and 82% within sets of described phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr) sites respectively for which only a single piece of identification evidence is available (the majority of sites in PSP). Overall, we estimated that ~56,000 Ser, 10,000 Thr and 12,000 Tyr phosphosites in the human proteome have truly been identified to date, which is lower than most published estimates. Furthermore, our analysis estimated ~91,000 Ser, 49,000 Thr and 26,000 Tyr sites that are likely to represent false-positive phosphosite identifications.

Conclusions

Researchers should be aware of the significant potential for false positive sites to be present in public databases and evaluate the evidence behind the phosphosites used in their research.

P13.20

Phospho-proteomic Analysis of Microbe-Associated Molecular Patterns (MAMPs) Signalling in Food Security

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Food security are important issues in the World because crops and vegetables have been suffering with serious challenges from various pests and diseases. The first layer in plant immunity to detect highly conserved components of microbes, such as flagellin and chitin, are called Microbe-Associated Molecular Patterns (MAMPs). The overlaps and differences among these MAMP signalling pathways remain unclear in plants such as Brassicas, maize, and tomatoes. Phosphorylation is an excellent post-translational modification to focus on because it can form the basis for physical enrichment of signal-transduction components and could be identified by high-throughput Mass spectrometry. Based on our newly established workflow including MAP kinases activation and phosphoproteomics, we have identified conserved phosphoproteins who changed their phosphorylation levels in plant defence among different species. Our project will draw a latest phosphoproteome map of the plant immunity and offer candidate genes to be used in genetic breeding, which will benefit our food and life very much.

P13.21

Towards Nanopore based Single-Molecule Bottom-Up Proteomics

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Introduction: Recent advances in single-molecule nanopore electrophysiology have led to single-molecule analyzers for the long-read sequencing of DNA. Advantageously, nanopore sensors can be incorporated in palm-sized devices, making them highly portable and economical to manufacture. However, the analysis of proteins using nanopores is complicated by the complex physicochemical structure of polypeptides. Here, we establish a technique for the detection of proteolytically cleaved proteins by the signal they induce once enclosed inside an engineered nanopore (1). We measure the conductance across the nanopore and have previously shown that this correlates with the mass of translocating peptides.

Methods: The Fragaceatoxin C (FraC) nanopore was engineered at its recognition interface by mutation of residue G13 to phenylalanine (G13F-FraC) (2). Protein digests were subjected to single-molecule nanopore electrophysiology using G13F-FraC. The current fluctuations observed of translocating peptides were characterized and shown separable based on their ion exclusion.

Results: The G13F-FraC is a sub-nanometer biological nanopore that allows cation-π interactions between the phenylalanine residue and positively charged N-terminal of translocating peptides (2). We show that G13F-FraC allows the rapid detection of a range of peptides in a manner analogous to (the early days of) mass spectrometry. The obtained signal from peptide translocation through the FraC nanopore shows a direct correlation between the occupied volume and the observed current blockade, allowing protein fingerprinting. Importantly, these sensors are amenable for the native detection and localisation of post-translational modifications (3).

Conclusions: We show that this system is capable of fingerprinting proteolytic protein digests in a way that is similar to conventional bottom-up proteomics, promising a next generation of fast and affordable protein analysers.

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P13.22

Representing Proteins and Peptides with Variational Feature Information in Graphs using ProtGraph

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Introduction
With ProtGraph, we provide a novel representation of proteins and digested peptides based on directed and acyclic graphs allowing fast access to all possible combinations of digested and sequentially varied peptides, and thus to identify spectra originating from such peptides. The SP-EMBL-Entries of UniProt KB not only provide the canonical sequence, but also additional feature-information (isoform-, variant-, initiator methionine- and signal peptide-information) of proteins which ProtGraph can utilize.

Methods
The features are added to the protein-graphs by modifying an initial protein-graph consisting of the canonical sequence by specific rules. Additionally, the graphs can be further modified with digestion information and computationally optimized. We extend ProtGraphs feature-parsing ability to additionally parse MUTAGEN and CONFLICT features from SP-EMBL-Entries, appending the information to the protein-graphs. Additionally we implement a amino acid replacement mechanism in ProtGraph, allowing to substitute amino acids in order to resolve ambiguous amino acids abbreviations.

Results
First, we illustrate the search space of peptides on complete UniProt, by counting the number of possible peptides contained in protein-graphs by using a dynamic programming approach. We show differences of the size of the search space by including/ignoring feature information and amino acid replacements. We illustrate that in some cases, the number of peptides can get unmanagably large, so that an export with all combinations in FASTA format is not possible. However, using smaller sets of variants, e.g. obtained by sequencing it is feasible to generate FASTAs and identify variants in mass spectra.

Conclusions
Protein-graphs are a good representation for proteins if feature information is important. The graph structure itself is compact while containing huge amounts of proteins/peptides. We are specifically interested in querying these graph by aribtrary information like mass/weight or containing peptide, to make a identification of all possible combinations of annotated features feasible.

P13.23

Unveiling New Proteoforms of the Industrial Workhorse Corynebacterium Glutamicum through Top-down Proteomics

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Introduction: The bacterium Corynebacterium glutamicum produces a variety of industrial relevant biomolecules, especially amino acids. Previous reports have evidenced metabolic regulation in C. glutamicum by post-translational modifications (PTMs) (1). Here, we applied top-down proteomics (TDP) to reveal putative C. glutamicum PTM-mediated metabolic regulation.
Methods: C. glutamicum ATCC 13032 intracellular proteins were submitted to Gel-Eluted Liquid Fraction Entrapment Electrophoresis (GELFrEE) fractionation (2). Proteins fractions below 50 kDa proceeded to LC-MS/MS and proteoforms´ identification was performed by TopPIC Suite (3).
Results: We could identify 5127 PrSMs, 1125 proteoforms and 273 proteins. Moreover, 177 proteins related to ribosome, pyrimidine metabolism, transmembrane helix and biosynthesis of amino acids were identified with mass shifts (Δm), suggesting the presence of PTMs. Important amino acids biosynthesis proteins and bacterial´s proteins secretion system were identified with Δm of 70 Da, 28 Da and truncations. Such modifications suggest unknown metabolic regulations in these pathways. Possible mechanisms of regulation could be degradation, inactivation, or protein-protein interaction disturbance.
Conclusions: TDP identified thousands C. glutamicum proteoforms with different PTMs, lightning the way to possible new mechanisms of regulation in the amino acid production, protein secretion system and translation of this bacterium.

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P13.25

Identification of Interaction Partners of Calcitonin Receptor-like Receptor in Primary Human Dermal Lymphatic Endothelial Cells.

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Introduction: The calcitonin receptor-like receptor (CLR) is a G protein-coupled receptor (GPCR) that is expressed in human skin and primary human dermal lymphatic endothelial cells (HDLECs). CLR-mediated signalling in the lymphatic system is implicated in some skin-related diseases, including lymphoedema and melanoma. However, the pharmacological properties of CLR expressed in human cells and the CLR-mediated signalling are poorly characterised. Consequently, CLR potential as a target for therapeutic intervention remains unclear. The aim of this study was to conduct a whole proteome profile analysis of HDLECs and identify potential protein interaction partners of CLR.
Methods: HDLECs were cultured to 80% confluency and lysed in radioimmunoprecipitation assay (RIPA) buffer, containing protease and phosphatase inhibitors. CLR was immunoprecipitated (IP) from total protein lysates using an in-house rabbit anti-human CLR polyclonal antibody (LN-1436) (1) captured with protein G magnetic beads. CLR depletion efficiency was examined by immunoblotting. All experiments were performed in quadruplicates. Total lysate samples were processed by single-pot, solid-phase-enhanced sample preparation (SP3) (2). Proteome profiling was carried out using the SP3-processed total protein digest or on-bead trypsin digestion of the IP samples, followed by label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Q-Exactive HF-X system. Data processing, bioinformatics and statistical analysis were conducted using the MaxQuant and Perseus software platforms.
Results: Immunoblotting experiments indicated complete depletion of endogenous CLR from whole cell lysates. Of 4919 proteins identified by LC-MS/MS analysis (FDR: 0.01) in primary HDLECs, 26 were considered as significantly enriched (FDR-adjusted p-value=0.005) and potential interaction partners of CLR.

Conclusions: Our study is the first to identify a cohort of potential binding partners for endogenously expressed CLR in primary HDLECs, together with a label-free quantitative proteomic profile of these cells.

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P13.27

Use of Proteomics to Study the Antifungal Effect of Metformin on C. albicans

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Introduction
The treatment of fungal infections is an important health problem, and the resistances appeared against the current arsenal of antifungals are increasing. Thus, the discovery of new targets or antifungal agents remains as an important task.
Metformin is a biguanide administered as a first-line treatment for Type II Diabetes Mellitus and it has been published as an anti-Candida drug, especially against C. glabrata, and with synergistic effect with other antifungals. Although metformin has been described as AMPK agonist, its mechanism of antifungal action remains elusive. Our studies on the effect of metformin have been done using C. albicans as main species causing invasive candidiasis.
Methods
C. albicans has been treated with increasing conditions of metformin up to 100mM. Several culture media (YPD with and without serum, RPMI, Spider) and conditions (30ºC and 37ºC) have been used.
The conditions for the proteomic study were 50mM of metformin, 6 h of treatment at 37ºC in RPMI medium and with 60 rpm of agitation to warrant a quality proteomic sample. The proteomic study has been done using the Labelfree technique and 4 biological replicas have been analyzed.
Results
100 mM Metformin causes growth inhibition, especially in RPMI at 37ºC, a decrease in the filamentation, in the adhesion and in the invasive growth, all of them phenotypes important for C. albicans virulence. To deepen into the antifungal mechanism of action, we have addressed the differential proteomic study. The analysis allowed the identification and quantification of 1899 proteins, 206 of them presenting differences in abundance due to metformin exposure. Of these, 127 increased and 79 decreased due to the action of the drug. The most relevant functions of these proteins are related to antifungal response, filamentation, biofilm formation and metabolism, being 9 essential proteins for the microorganism that could be new antifungal targets.

P13.28

Toward Better Pre-clinical Sarcoma Model Using Decellularized Extracellular Matrix

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Introduction: In vitro models are crucial tool for the pre-clinical research. However, the assessments using in vitro models (for example, drug screening) do not necessarily reflect the clinical results. To improve this issue, researchers pay attention to the decellularization that is a method to remove cells from tissue and leave extracellular matrixs (ECMs) components. The advantage of decellularized tissue is to contain similar ECMs components to the original tissue. ECMs extracted from decellularized tissue, that we call dECMs, are used as coating material on the surface of cell culture plates. dECMs are now utilized for the research in oncology. Although not only epithelial tumors but also sarcomas are regulated their pathway related to growth, invasion and apoptosis by ECMs, there are limited reports that investigate the effect of dECMs on the sarcoma cells. In this study, for the improvement of pre-clinical model with dECMs, we investigated the effect of dECMs on the sarcoma cells and fabricated the coating material of dECMs derived from sarcoma cell line.
Methods: To accomplish the purpose, we performed the decellulatization and digestion of mice tissues. After the digestion, we obtained the dECMs and sought the components via proteomic analysis using gel electrophoresis. Osteosarcoma cells were seeded on the dECMs coating cell culture plate to reveal the effect on cell proliferation and migration. To acquire dECMs from osteosarcoma, we fabricated spheroids of the cells.
Results: Through the in vitro assays and proteomic analysis with dECMs, we found that dECMs had potential to address the challenges of in vitro cell culture of sarcoma. Furthermore, we successfully decellularized the spheroids of osteosarcoma cells with detergent.
Conclusions: After the sufficient examination of decellularized method with cell line, our next work is to fabricate the dECMs derived from patient' biopsy sample of osteosarcoma for the further improvement of pre-clinical study.

P13.30

1DE Gel-Concentration Procedure for LC-MS/MS Analysis of Sds-Extracts of Human Chorionic Villus

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Introduction: For efficient solubilization of proteins from solid tissues, the high concentrations of detergents typically required, but they may inhibit trypsin activity and suppress LC-ESI-MS ionization (1). Therefore, the removal of the detergent is very important for the subsequent in-depth profiling of the proteome and this stage is mandatory in the sample preparation workflow. Our study was aimed to determine whether the processing of SDS-extracted protein samples using polyacrylamide stacking gel (4%T) prior to protease digestion is suitable for sample preparation in proteomics.
Methods: 2% SDS-containing extract of chorionic villus was deposited onto three gel runs in an amount of 50 µg of total protein per line. 1DE-gel concentration was carried out for 40-50 minutes at 50 V, resulting single protein band was used entirely for in-gel digestion and LC-MS/MS analyzing.
Results: About a hundred low abundance (CVNSAF < 0.16) proteins have been identified using SearchGUI with simultaneous integrated search algorithms X!Tandem and MS-GF+. Our analyses mapped the proteins that were not previously detected in trophoblastic cells according the Human Protein Atlas. Moreover, we successful to find out pregnancy-specific beta-1-glycoprotein 7 (PSG7) which the existence is unsure ("protein uncertain") according to neXtProt human protein-centric knowledge platform. We managed to register 8 peptides that matched PSG7 among which one peptide 256DVSTFTCEPK was unique. IdentiProt identification based on the open-source IdentiPy algorithm (2) allowed us to additionally detect a second PSG7-specific peptide 91YGPAYSGR.
Conclusions: The results indicated that 1DE-concentration procedure coupled with in-gel digestion, LC-MS/MS and combinational usage of different bioinformatics tools could yield excellent depth of analysis in a single protein band and effectively ascertain low abundance (CVNSAF ≤ 0.16) and missing proteins in 2% SDS chorionic villi extracts.

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P13.31

Characterization of Biological and Metabolic Responses to PH Changes in Staphylococcus Epidermidis

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INTRODUCTION: Staphylococcus epidermidis is a Gram-positive commensal bacterium found in human skin. It is considered an opportunistic pathogen associated with nosocomial infections. Indeed, S. epidermidis is the main cause of coagulase negative staphylococci infections associated with medical devices.
METHODS: We studied the impact of pH conditions which mimic the skin pH (5.5) and blood pH (7.4) in a S. epidermidis commensal strain (19N) recurring to proteomics, by nanoscale liquid chromatography-tandem mass spectrometry (nano LC-MS/MS) and NMR-based metabolomics of cell extracts.
RESULTS: The proteomic results show that pathways related with energy production like glycolysis/gluconeogenesis, TCA cycle, butanoate metabolism as well as transport systems or proteins related to bacterial virulence are differentiated among the studied conditions. By NMR we were able to quantify forty-five metabolites, being choline, sn-glycero-3-phosphocholine, cystathionine, asparagine, aspartate, lactate and tyrosine the most discriminatory among experimental conditions. Pathway analysis from quantified metabolites reveal that the more relevant and impacting pathways were glycerophospholipid metabolism, glycine, serine and threonine metabolism, nitrogen metabolism, β-alanine metabolism, arginine and proline metabolism.
CONCLUSION: This study indicated that S. epidermidis 19N adaptation to the blood pH rely on the increase of glycolysis/gluconeogenesis, TCA cycle, pyruvate metabolism and purine synthesis, while the glycerolipid and glycerophospholipid metabolism and betaine biosynthesis is decreased.

P13.32

Proteomic analysis of mouse hearts treated with rattlesnake venom revealed modulation of proteins associated with mitochondria and cardiomyopathies

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Introduction: Crotalus durissus terrificus rattlesnake is the most lethal and it is the second cause of snakebite accidents in Brazil. Its venom is known to be cardiotoxic, neurotoxic, myotoxic, and nephrotoxic, being responsible for several disturbances for the affected individuals. In this study, we focused on the evaluation of the cardiotoxic effects of rattlesnake venom on mice heart at different time points after venom injection and analyzed the protein profile using high-resolution mass spectrometry-based proteomics analysis and histology analysis.
Methods: We injected 0.5 LD of C. d. terrificus venom on the gastrocnemius muscle and dissected the hearts 1 h, 6 h, 12 h and 24 h after venom injection. Proteins were lysed, chemically modified (reduced and alkylated) and digested with trypsin. Tryptic peptides were analyzed using an Ultimate 3000 nLC coupled to a Q-Exactive HF high-resolution mass spectrometer. Generated data were analyzed using Peaks, Perseus, Webgestalt, String and Cytoscape bioinformatics tools in order to identify, quantify, analyze and profile the GO of protein groups and protein-protein interactions.
Results: We were able to identify >1300 proteins in all conditions and observed that several proteins showed abundance changes over the time after venom treatment. Several of these proteins are related to mitochondria and mitochondrial pathways and heart diseases such as OPA1, SODM, MYG, MLRV, TNNT2, NDUS6, MYOZ2, CAVN4, PDLI5, and MYH6.
Conclusions: The toxic effect of venom affected several proteins that perform different functions in the heart tissue, triggering different immunological and biochemical effects triggering disturbances from the cellular to physiology and structure of the heart from early to late time points.

P13.33

Proteomic Analysis to Identify Candidate Biomarkers Associated with Skin Co-exposure to Ultraviolet Radiations and Benzo[A]Pyrene

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Human skin, and mainly the outer epidermis, is continuously exposed to environmental stressors, mainly air pollutants. Air pollutants can significantly impair, via oxidative damage, the normal functioning of human skin's proteins, lipids and/or nucleic acids, thus triggering different skin disorders including aging, psoriasis, inflammatory reactions and skin cancer. Among air pollutants that can harm the skin are ultraviolet radiations (UVR) and Polycyclic aromatic hydrocarbons (PAHs ; such as Benzo[a]pyrene). Exposure to UVA has been associated with skin aging whereas UVB alone could account for sunburn. UVA, along with UVB, could trigger photoimmunosuppression and development of different cutaneous cancers (photocarcinogenesis). Exposure to BaP could trigger different pathologies including hepatotoxicity, neurotoxicity, immunotoxicity and placental toxicity. Intriguingly, skin tumor incidence increases in response to BaP + UVA treatment, compared to BaP- or UVA-treatment alone. Till date, a detailed analysis of the biological processes that are altered following coexposure of human skin to BaP + UVR has not been performed. In this study, we used a mass-spectrometry-based proteomic analysis to identify differentially expressed proteins in human epidermal skin cells being exposed to either no pollutant, UVR alone, or UVR + BaP. Accordingly, we identified a number of proteins that could serve as potential biomarkers of skin coexposure to UVR + BaP.

P13.34

High-throughput Lipidomics using
Ion-mobility enhanced DDA and DIA Mass Spectrometry

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Introduction: The quantitative analysis of small molecules contained within complex mixtures represents a challenging problem in analytical chemistry. Over the last decade, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been established as the gold standard method for robust and unbiased analysis of complex mixtures. However, current gold standard targeted metabolomics methods using MRM/PRM are limited in analyte throughout.
Methods: We propose the development of a novel MS method based on data-independent acquisition (DIA/SWATH-MS) and ion mobility separation, which would allow us to increase the analyte throughput from dozens of analytes to thousands of analytes using targeted metabolomics.
Results: First, we optimized data acquisition parameters using the NIST Tandem MS/MS dataset to maximize the number of unique ion signatures. Our simulations show that DIA outperformed MS1-only and MRM-based methods with regards to specificity by a factor of ~2.8-fold and ~1.8-fold respectively. Next, we experimentally optimized data acquisition parameters to develop a novel data-dependent acquisition MS method coupled to an ion mobility device (timsTOF Pro, Bruker Corporation), providing an additional dimension of separation with increased specificity and analyte throughput. Ion mobility (DDA-IM) improves the coverage of lipid classes with a ~2-fold increase in lipid annotations. Using this method, we will generate a library of accurate MS coordinates and enhance the sensitivity and selectivity of this acquisition approach by selecting optimal collision energies for each DIA window and isolation window settings using the SRMCollider software. Finally, I will apply the developed method to study longitudinal analyte variation in human plasma, a major problem in quantitative metabolomics, especially in applications of toxicology (pesticides), forensics and systems biology.
Conclusions: This will further our understanding of functional interactions in the complex metabolic consortia and how these interactions enable the central application of life.

P13.35

Establishment and Characterization of a Novel Cell Line, NCC-MPNST6-C1, Of Malignant Peripheral Nerve Sheath Tumor

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Introduction: Malignant peripheral nerve sheath tumor (MPNST) is a rare subtype of soft-tissue sarcomas, being defined as nerve sheath tumors arising from a peripheral nerve. MPNST is an aggressive tumor with a poor prognosis. After a curative surgery, the local and distant metastasis was observed in more than half of patients with MPNST. The effective adjuvant chemotherapy has not been established yet, and more research will be required for better prognosis in MPNST. While the patient-derived cell lines are critical for pre-clinical studies, only a few cell lines of MPNST are available from public cell banks. Thus, we aimed to generate novel cell lines of MPNST in this study to identify effective anticancer agents.

Methods: Using surgically resected tissues, we established the cell line and designated it as NCC-MPNST6-C1. The donor patient was a 72-year-old man and the tumor was located on the right thigh. First, STR allele pattern analysis by capillary electrophoresis was performed to authenticate NCC-MPNST6-C1 cells. Single nucleotide polymorphism (SNP) array genotyping was then performed on NCC-MPNST6-C1 cells to examine for chromosomal aberrations. Spheroid formation and invasion ability were also examined. In addition, screening of 214 anticancer agents was performed to identify anticancer agents with growth inhibitory effects on NCC-MPNST6-C1 cells.

Results: NCC-MPNST6-C1 cells showed STR allele patterns similar to those of the original tumor, and exhibited chromosomal abnormalities. They proliferated more than 35 passages in 5 months. The doubling time was approximately 75 hours. The cells had capabilities for spheroid formation and invasion. Furthermore, we identified anticancer agents that had growth inhibitory effects on NCC-MPNST6-C1 cells.

Conclusion: We established a novel cell line of MPNST and designated it as NCC-MPNST6-C1. Through a series of characterizations, we proved a utility of this cell line for in vitro study, especially in drug screening.

P13.36

Developing a Pipeline for Isoform-Level Multi-Omics Data Analysis

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Inbred strains like C57 and DBA have shown considerable differences in physiology and behavioural pattern, hence are extensively used in neuroscience research. We postulate that DNA-methylation may play a role in the behaviour differences in the two inbred strains and the correlation analysis will allow to understand the impact of region-specific DNA methylation. We have selected a steady-state mouse model and analysed transcriptome using both RNA-seq and Iso-Seq, methylome using BS-seq, and proteomics using SWATH-MS on the brain-cortex of 10 weeks old male mice (n=4). We employed a proteogenomic approach to identify the expressed isoforms in the brain cortex. We used Iso-Seq as reference for SWATH-MS and RNA-seq identification and quantitation of isoforms and corroborated the differences in the strains with the differentially methylated regions (DMRs) in the expressed isoforms. We identified isoforms of Padi2 and Me2 with differential expression to harbor DMRs in their exonic and intronic region. Literature search on Padi2 and Me2 associates them to brain disorders like Alzheimer's and Epilepsy. Me2 is also related to audiogenic seizure susceptibility, a known behaviour difference in the two mice strains. We developed a multi-omics pipeline for isoform-level analysis. These preliminary findings illustrate potential benefit of the developed pipeline for future behavioural studies.

P13.37

Metabolomic Analysis of Amniotic Fluid Samples Infected by Zika Virus: Microcephalic versus Non-microcephalic Fetuses

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Introduction: Zika virus (ZIKV) can be transmitted vertically to the fetus during pregnancy and cause Congenital Zika Syndrome (CZS). The fetuses infected in the first trimester of gestation presents higher chances to develop the syndrome. Several studies have shown that ZIKV impairs neurodevelopment causing microcephaly, a severe reduction of the brain. However, the molecular differences between microcephalic and non-microcephalic phenotypes that result from ZIKV infection are poorly understood. Here our main goal was to identify the metabolic pathways dysregulated in ZIKV infected amniotic fluid (AF) and its relation to CZS through metabolomic analysis.

Methods: AF samples were analyzed by untargeted metabolomics. Metabolites previously extracted with methanol were analyzed in a Q Exactive Plus (Thermo Scientific) mass spectrometer coupled to a UHPLC (Ultimate 3000, Thermo Scientific). We analyzed seven AF: three from healthy women (CTR group) and four from ZIKV infected patients bearing non-microcephalic and microcephalic fetuses (Z+ and MC+ groups, respectively). Compound discoverer software (version 3.2) was employed for metabolite identification and quantification. Statistical and functional analyses were performed in MetaboAnalyst (version 5.0).

Results: A total of 243 metabolites allows us to differentiate the groups under investigation. Infected patients (Z+ group) were characterized by glycerophospholipid metabolism impairment, which was reflected in the decreased concentration of several glycerophosphocholines and glycerophosphoethanolamines. In contrast, some hydroxy fatty acids, fatty esters, and dicarboxylic acid were up-regulated. These findings are coherent with previous reports of lipid metabolism manipulation by flaviviruses, extremely important for their replication cycle. Interestingly, microcephalic phenotypes presented a higher decrease in glycerophospholipid abundance compared to the Z+ group.

Conclusions: ZIKV impair negatively the glycerophospholipid metabolism. These findings suggest that an accentuated decrease in glycerophospholipid concentration can impact brain development. This study contributes to the understanding of CZS pathology and to discover potential biomarkers for CZS prognosis in the early stages of pregnancy.

P13.38

GSH Mediated Alleviation of AAL Induced Stress in Plants- A Proteomic Approach in Solving the Cryptex of Plant Stress Signaling

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Introduction: The role of glutathione (GSH), in plant defense is an established fact. However, its mechanism of interaction with other stress modulators is yet to be explored in-depth. AAL toxin, the major virulent effector molecule produced by Altenaria alternata f. sp. Lycopersici, can affect several species of economically important plants. The present study has been an effort to understand how GSH interacts with other phytoprotectants and modulating stress signaling in favour of the plants, under necrotrophic attack induced by AAL.
Method: Arabidopsis leaves (wild type Col-0 and transgenic AtECS1 exhibiting enhanced GSH) were harvested from both control and AAL treated Col-0 and AtECS1 for subsequent studies. An initial proteomic analysis was performed using nano LC–MS/MS of all four plant samples. Following the identification of several protein species, a few among them were selected for further studies using qRT-PCR along with western blotting and HPLC, to validate and understand the mechanism of stress tolerance.
Results: Functional categorization following identification revealed that a significant number of proteins, belong to stress and defense category. Among these some proteins were found to be salicylic acid (SA) and ethylene (ET) responsive. Following this trail, relative expression levels of the corresponding genes of the identified protein species influenced by SA and ET were checked, along with few other stress responsive genes, known to confer resistance. It was found that the above-mentioned genes, were upregulated in AAL treated AtECS1 compared to Col-0, while, the genes related to ET were down regulated in AAL treated AtECS1 compared to Col-0. Similar trend is followed while the endogenous SA and 1-aminocyclopropane-1-carboxylate (ACC) levels were checked along with the proteins influenced by both SA and ET.
Conclusion: GSH promotes AAL induced stress tolerance through SA mediated suppression of ET besides influencing several other stress modulators in favour of the plants.

P13.39

Dried Blood Spot as a Biomarker Source: A Bridge between Proteins and Metabolites in the Omics Era

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Introduction. Dried blood spot (DBS) samples are the newest field of research interest due to their applicability to clinical diagnostics and ease of handling by minimal risk of contamination, limited volume, zero invasiveness for collection and possibility of long-term storage [1]. Beyond the use for expanded newborn screening, DBS samples caught the eye of untargeted metabolomics approaches for biomarker discovery [2]. In this context, proteins from DBS could likewise be useful to achieve screening biomolecular profiles by mass spectrometry (MS) analysis on easily accessible samples, thus providing a novel clinical tool to be combined with other omics-based technologies. Methods. DBS samples from one cholesteryl ester storage disease (CESD) patient and its healthy control (HC) were extracted and tryptic digested by filter-aided sample preparation (FASP) for shotgun proteomics; other DBS samples from CESD and HC were in parallel extracted and analyzed for untargeted metabolomics. Proteomics and metabolomics were performed by nano-LC-Orbitrap-MS in data-dependent acquisition (DDA) mode and the identified compounds (proteins and metabolites) with differential expression were combined together for functional analysis on Ingenuity Pathway Analysis. Results. DBS proteomics by FASP allowed to ensure quantitative reproducibility, detergent-free sample preparation and clean peptides elution. DBS untargeted metabolomics revealed thousands of compounds by acquisition in positive and negative ionization modes. Pathway analysis of differential proteins and metabolites taken together highlighted a specific modulation of molecular networks related to steroids, sphingolipids and fatty acids metabolism in CESD. Conclusions. The conjugation of DBS proteomics and metabolomics can favour biomarker discovery for the study of rare disorders, such as lysosomal storage diseases, thus facilitating their underlying mechanisms comprehension. This combined approach can unfold new roads from discovery to clinical use. Lipid pathway alterations deserve further molecular validation.

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P13.40

The Metaproteomics Initiative: Coordinating International Efforts for Propelling the Functional Characterization of Microbiomes

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Microbial communities play a major role in biogeochemical cycles as well as in human health and disease. For example, the human gut microbiome has important roles in digesting food and modulating host immunity. To understand how microbiomes function and how they interact with the host, metaproteomics can provide unique insights because of its role in connecting genomic and metabolic information[1]. This field has therefore gained an increasing interest over the past decade and numerous innovations are anticipated in the near future.

A growing community of metaproteomics researchers had the opportunity to meet at several international symposia since 2016, and launched several training sessions and even an interlaboratory comparison: the CAMPI study[2]. To develop standards and promote education in this field, we established the Metaproteomics Initiative, an international community that currently brings together over 90 members from over 47 research groups from 17 countries. This Initiative aims to promote dissemination of metaproteomics fundamentals, advancements, and applications through collaborative networking in microbiome research. It aims to be the central information hub and open meeting place where newcomers and experts interact to communicate, standardize, and accelerate experimental and bioinformatic methodologies in this field.

We hereby also invite the entire (meta)proteomics community to join this Initiative and discuss potential synergies at the interfaces with other disciplines, and to collectively promote innovative approaches to gain deeper insights into microbiome dynamics. More information can be found on our website (https://metaproteomics.org/) and via our Twitter account (@MetaP_Init).

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P13.42

Glycoproteomic Study of Saccharomyces Cerevisiae Yeast Cell Wall Mannoproteins Reveals a Dynamic Molecular Change Depending on Culture Strategy and Conditions

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Introduction: Yeast cell wall (YCW), the outermost organelle of the yeast cell, is composed of an inner polysaccharide layer, consisting of β-glucans majorly cross-linked to a minor amount of chitin, to which are bound mannoproteins. YCW mannoproteins have functional and health promoting properties related to their particular molecular structure, and their composition is suggested to vary depending on environmental conditions, but have been little investigated. This work aims to prove YCW mannoproteins dynamic change at the molecular level using mass spectrometry (MS).

Methods: S288C yeast strain was cultured in YPD medium in bioreactors following batch and fed-batch strategies. YCW, obtained by mechanical disruption, were subjected to an ultracentrifugation using an iodixanol continuous density gradient (18-48%). The resolved band were O-deglycosylated chemically or enzymatically and N-deglycosylated by PNGase F/Endo H (20 U) in an adapted eFASP method. The resulting peptides were analyzed by nanoESI-LC-MS/MS. Proteins were identified using Proteome Discoverer 2.2 against SGD S288C dataset. O- and N-glycans were chemically derivatized by aminative reduction reaction or a newly developed miniaturized permethylation and subsequently analyzed by µLC-MS and CE respectively.

Results: We showed the reliability of the YCW extraction and ultracentrifugation methods for yeast cell wall enrichment. In addition, the proteins profiles differ qualitatively and quantitatively depending on growth phase and culture mode, and we identified some of their protein markers. Mannoproteins O- and N-glycans were isolated simultaneously and efficiently permitting their analysis by MS and CE respectively following their chemical derivatization. The released O-glycans were detected by mass spectrometry coupled to RPLC upon their derivatization with ABBE. CE has allowed the separation of APTS-derivatized N-glycans, whereas their permethylation and subsequent analysis by mass spectrometry has allowed their identification containing up to 12 mannoses.

Conclusions: This work describes the first one-pot glycoproteomic methodology revealing YCW glycoproteomic change depending on culture strategies.

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