EuPA
EUROPEAN PROTEOMICS ASSOCIATION

Deutsche Gesellschaft für Proteomforschung e.V.

Abstract booklet

Proteomic Forum | EuPA 2022

3-7 April 2022 | Leipzig, Germany

Invited lectures

IL01

Pathology from the subcellular scale on up

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High parameter single cell analysis has driven deep understanding of immune processes. Using a next-generation single-cell "mass cytometry" platform we quantify surface and cytokine or drug responsive indices of kinase target with 45 or more parameter analyses (e.g. 45 antibodies, viability, nucleic acid content, and relative cell size). Similarly, we have developed two advanced technologies termed MIBI and CODEX that enable deep phenotyping of solid tissue in both fresh frozen and FFPE formats (50 – 100 markers). Collectively, the systems allow for subcellular analysis from the 70nm resolution scale to whole tissue in 3D.

I will present evidence of deep internal order in immune functionality demonstrating that differentiation and immune activities have evolved with a definable "shape". Further, specific cellular neighborhoods of immune cells are now definable with unique abilities to affect cellular phenotypes—and these neighborhoods alter in various cancer disease states. In addition to cancer, these shapes and neighborhoods are altered during immune action and "imprinted" during, and after, pathogen attack, traumatic injury, or auto-immune disease. Hierarchies of functionally defined trans-cellular modules are observed that can be used for mechanistic and clinical insights in cancer and immune therapies.

IL02

Improving proteome coverage of single cells

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Technological advances have started to push single-cell analysis in the realm of proteomics, which is an important development to start addressing questions that cannot be investigated in bulk populations, such as cellular heterogeneity. Multiple challenges are still to be met in sample preparation, MS acquisition and data analysis to improve sensitivity and proteome coverage, requiring the redesign of several components in the pipeline as we know it for bulk proteomics. Although TMT labeling currently is the prevailing method for (multiplexed) single cell proteomics, alternative approaches deserve exploration to establish if true single-cell proteomics may become a reality.

Here we will present efforts from our lab to develop strategies for single-cell analysis by label-free proteomics. This is aimed at simplified and rapid sample processing to avoid protein losses, and at tailoring mass spectrometric analysis for low-input samples using timsTOF mass spectrometry. In addition, we recognize that high rates of missing values remain a serious problem in proteomics, which is even more severe in sparse single-cell data. To improve this situation for data-dependent MS acquisition, we have developed a novel data analysis workflow termed IceR, which uses direct ion current extraction (DICE) to transfer peptide identities across samples, and which quantifies peptide abundance even if initial peak detection failed. Implementing this in conjunction with robust feature-based identity propagation, IceR demonstrates superior quantification precision and data completeness compared to other quantitative workflows. Applied to single-cell proteomics data, IceR enhanced the number of reliably quantified proteins, improved discriminability between single-cell populations, and allowed reconstruction of a developmental trajectory. Collectively this indicates that combined improvements in sample preparation, MS acquisition and data analysis promise a bright future for single-cell proteomics.

IL03

Exploring functional protein covariation across single cells

N. Slavov[1]

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Biological functions are reflected in the natural variation of proteome configurations across individual cells. Emerging single-cell proteomics methods may decode this variation and empower inference of biological mechanisms with minimal assumptions. I will describe both established and emerging single-cell mass-spectrometry methods, and how these methods have allowed us to interpret protein covariation in different biological systems, including primary macrophages and melanoma cells. The focus of my talk will be on conceptual innovations and strategies for

data acquisition and interpretation that make single-cell protein analysis accessible, robust and highly quantitative.

IL04

New Developments in Mass spectrometry based Single-Cell Proteomics

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The analysis of single cell proteomes has recently become a viable complement to transcriptomics and genomics studies. Proteins are the main driver of cellular functionality and mRNA levels are often an unreliable proxy of such. Therefore, the global analysis of the proteome is essential to study cellular identities. Both multiplexed and label-free mass spectrometry-based approaches with single cell resolution have lately attributed surprising heterogeneity to believed homogenous cell populations. Even though specialized experimental designs and instrumentation have demonstrated remarkable advances, the efficient sample preparation of single cells still lacks behind. Here, we introduce the proteoCHIP, an universal option for single cell proteomics sample preparation at surprising sensitivity and throughput. The automated processing using a commercial system combining single cell isolation and picoliter dispensing, the cellenONE®, allows to reduce final sample volumes to low nanoliters submerged in a hexadecane layer simultaneously eliminating error prone manual sample handling and overcoming evaporation. The specialized proteoCHIP design allows for the direct injection of single cells via a standard autosampler resulting in around 1,500 protein groups per analytical run at remarkable reporter ion signal to noise while reducing or eliminating the carrier proteome. We identified close to 2,600 proteins across 170 multiplexed single cells from two highly similar human cell types. This dedicated loss-less workflow allows to distinguish *in vitro* co-differentiated cell types of self-organizing cardiac organoids based on indicative markers across 150 single cells. In-depth characterization revealed enhanced cellular motility of endothelial cells and acute myocardium sarcomere organization in cardiomyocytes. Our versatile, and automated sample preparation has not only proven to be easily adoptable but is also sufficiently sensitive to drive biological applications of single cell proteomics.

IL05

Crosslinking mass spectrometry in the age of AlphaFold

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Crosslinking mass spectrometry is now a well-established experimental technique for investigating protein interactions, structure and function. Once considered an outdated technology in the midst of the resolution revolution of electron microscopy (EM), it turned into an indispensable tool in single particle cryo-EM studies to illuminate areas with missing structural information due to flexibility. Crosslinking MS remains unparalleled in its ability to provide structural information in complex systems.

Artificial intelligence program-based predictions of protein structure (e.g. by AlphaFold) are the most recent revolutionary advancement in structural biology with wide-ranging impacts that are yet to be fully understood. To understand how this approach complements crosslinking MS we will delve into different aspects of AlphaFold and crosslinking, taking a look at false positive rates, false negative rates, model prediction, model selection, model validation and the scale and dynamics of protein-protein interfaces. Ultimately, our structural understanding of biology stands to gain much from combining AlphaFold and crosslinking data, particularly in our drive towards building a structural and functional understanding of the cell.

IL06

The use of sebum to determine biomarkers of Parkinsons disease, following the nose for earlier diagnosis and non-invasive sampling

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Parkinson"s disease (PD) is the second most common neurodegenerative disorder and identification of robust biomarkers to complement clinical diagnosis will accelerate treatment options. Increased oiliness and flaky skin, especially on the face and scalp, is a common symptom of PD, first noted by Krestin in 1927. The light yellow, oily substance present on all human skin, is known as sebum, and increased sebum production is a hallmark of PD. We have previously shown that sebum contains volatile biomarkers of PD, and that it can reveal mitochondrial dysregulation as PD progresses. Here we set out to develop a method to analyse sebum in its native state to facilitate rapid assessment of PD status.

Methods

We demonstrate the use of multiomic MS methods for PD diagnosis and prognosis. We also show direct infusion of sebum from skin swabs using paper spray ionisation coupled with ion mobility mass spectrometry (PS-IM-MS) to determine the regulation of molecular classes of lipids in sebum that are diagnostic of PD. A semi quantiative PS-IM-MS method for sebum samples that takes three minutes per swab was developed and optimised. A subset of the statistically significant molecules has DT resolved features only observed in PD samples, and we focus on their elucidation.

Preliminary Data

The multiomic methods have been applied to skin swabs collected from 650 people and we have elucidated ~ 5000 features from LC-MS and c. 500 from TD-GC-MS from each subject which were independently analysed.

For PS IMS-MS data, to annotate statistically important features in the DT *vs. m/z* spectra we employed accurate mass searching of available databases (HMDB and LipidMaps). These resources do not include experimental validation for lipids above mass 1400 Da, and provide only scant information for those above 700 Da. Focussing first on the singly charged ions in the range *m/z* 700-950 enabled tentative identification of multiple classes of lipids with the highest confidence assignment as triacylglycerides, CnHmOp, where n= 45-57 and m = 84-104 and p= 6 over a mass range of 700 to 950 Da.

Further annotation of these data used tandem MS as well as information from the ion mobility separation, *i.e.* CCS, of the detected species, this allows us to confirm and also to rule out putative identifications of lipid class.

In the higher mass envelope peaks differing by 14 Da have been assigned as a recurrent lipid series each changing by a single -CH2 unit. This would implicate the presence of lipids with an odd number fatty acid carbon chains, which are uncommon in healthy serum but have been previously reported to be present in sebum, as well as indicators of a disease state.

We will also present data from prodromal PD patients.

The implications of this method and approach for non-invasive sampling in clinical settings will be discussed, as well as the opportunity for MRM analysis of PD diagnostic biomarkers.

IL07

Quantifying the assembly of ultra-heterogeneous protein complexes

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We have been developing and applying mass-spectrometry-based approaches to interrogate directly the structure and dynamics of proteins. Here I will focus on the insights this has enabled in studying the evolution of specificity in assembly of molecular chaperone proteins, and how they interact with target proteins under the application of force. I will also present mass photometry, a new method we have

developed that allows the quantitative, label-free interrogation of proteins in solution2. The combination of mass measurement approaches provides an unprecedented opportunity to quantify the heterogeneity of protein assembly, elucidate the influence of the proteins" physical chemistry on their evolution.

IL08

Decoding the Protein Composition of Intact Nucleosomes and the Entire Human Proteome

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[2]*University of Chicago, Chicago, IL, United States*

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The posttranslational modifications (PTMs) decorating the four core histones in an intact nucleosome encode nuclear effectors that trigger defined cellular events critical to health and disease. To investigate the composition of intact nucleosomes, we created a mass spectrometry (MS) based method that runs in native mode preserving non-covalent interactions for detection and top-down fragmentation of bioassemblies. The new approach named NucMS neither digests nor denatures nucleosomes and consists of three stages of tandem MS capturing the entire proteoform composition of intact nucleosomes. Nuc-MS was used to quantify changes in total mononucleosomes versus those immuno-enriched for histone H3.3, revealing 6-15 fold increases in histone forms elevated in euchromatin (like H2A.Z and H3K79me2). Histones are only one example of the proteoform diversity observed in the human body. The complete knowledge of proteoform structure and properties is fundamental to deciphering basic and translational research functions. Therefore, achieving the ambitious Human Proteoform Project (HPfP) aims to close this gap by developing and applying new technologies to reveal human biology"s molecular complexity and advance protein-level technology.

IL09

Expanding the Role of Proteoforms in the Field of Infectious Disease

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In past years, various examples have showcased the added value of addressing proteoforms in the field of infectious disease. For instance, for *N. meningitidis*, which is the etiologic agent of the cerebrospinal meningitis, a specific proteoform of the PilE protein, which plays an important role in bacterial virulence, was found tightly associated with crossing of the epithelial barrier and access to the blood stream.[1] For the same protein, highly glycosylated proteoforms were obtained from patients with evidence of meningitis and linked to immune escape.[2] More recently, O-mycoloylation of membrane proteins (porins) of *Corynebacterium glutamicum* was found to be a bone fide signal to direct the modified protein to bind to mycomembrane.[3] In clinical microbiology, proteoforms can also be used to differentiate bacterial strains that cannot be discriminated with MALDI-TOF MS, the technique used in routine in many hospital settings for the rapid identification of bacterial pathogens.[4] This talk will review a few of these examples and describe the top-down proteomics (TDP) workflows optimized for these applications, as well as new software tools developed to ease the analysis of TDP data.[5, 6] Perspectives will be discussed.

1. Chamot-Rooke, J. *et al.* Posttranslational Modification of Pili upon Cell Contact Triggers N. meningitidis Dissemination. *Science* **2011,** *331* (6018), 778-782.
2. Gault, J. *et al.* Neisseria meningitidis Type IV Pili Composed of Sequence Invariable Pilins Are Masked by Multisite Glycosylation. *Plos Pathog* **2015,** *11* (9).
3. Carel, C. *et al.* Identification of specific posttranslational O-mycoloylations mediating protein targeting to the mycomembrane. *Proc Natl Acad Sci U S A* **2017,** *114* (16), 4231-4236.
4. Dupre, M. *et al.* Optimization of a Top-Down Proteomics Platform for Closely Related Pathogenic Bacterial Discrimination. *J Proteome Res* **2021,** *20* (1), 202-211.
5. Dhenin, J. *et al.* TDFragMapper: a visualization tool for evaluating experimental parameters in top-down proteomics. *Bioinformatics* **2021**.
6. Lima, D. B. *et al.* ProteoCombiner: integrating bottom-up with top-down proteomics data for

improved proteoform assessment. *Bioinformatics* **2021,** *37* (15), 2206-2208.

IL10

Where is native MS ? Native MS is everywhere ! Overview of recent progress in native MS

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Mass spectrometry is generally understood as "molecular mass spectrometry" with multiple applications in biology (proteomic approaches, recombinant protein and monoclonal antibody characterization). Its application in so-called "native conditions" has been introduced 30 years ago to perform analysis of non covalent assemblies. Native mass spectrometry (nMS) has considerably evolved over the past ten years and is now admitted as a valuable technique to address stoichiometry issues along with dynamics of macromolecular complexes formation.

Here I will present an overview of the technological improvements in native MS and the diversity of applications on selected topics in biology.

IL11

Control of homeostasis in plant proteomes

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The proteomes of plants work through thousands of enzymes, structural, signaling and regulatory components to provide the air we breathe, the food we eat, the clothes we wear and many of the sustainable materials we rely on to build things. So proteomes of plants are important to us all. However these proteomes are not static, rather protein age, abundance, and functionality are impacted by the balance of protein synthesis and protein degradation to define proteostasis.

METHODS: Mass spectrometry-determined rates of protein degradation and synthesis provide a window into this process. They show degradation rates vary over orders of magnitude for different protein types in various cell types in different species. As a consequence, some proteins are continuously renewed and thus essentially "always new" in cells, while others accumulate across part or the whole history of living cells. The cellular cost of renewal is a substantial part of its energy budget in plants; typically 20-30% of cellular ATP is used in this process.

RESULTS: We will present new information on the way proteostasis is achieved in cereal grains, a major source of dietary protein, and how more than 25% of proteins synthesised in grain during development are degraded before grain maturation. We will highlight new data on changes to leaf protein turnover patterns during everyday events for plants – the diurnal pattern of day and night function (Figure 1) and the impact of high light intensity that damages a selective subset of leaf proteins (Figure 2). We will also discuss the impact of the age of plant proteins on their post-translational modification status and rate of replacement.

CONCLUSIONS: Dynamic changes in protein degradation process in plants ensure removal of older, damaged proteins and set the standing proteome but act within changing energy constraints and in responses to environmental and development stimuli.

Fig. 1

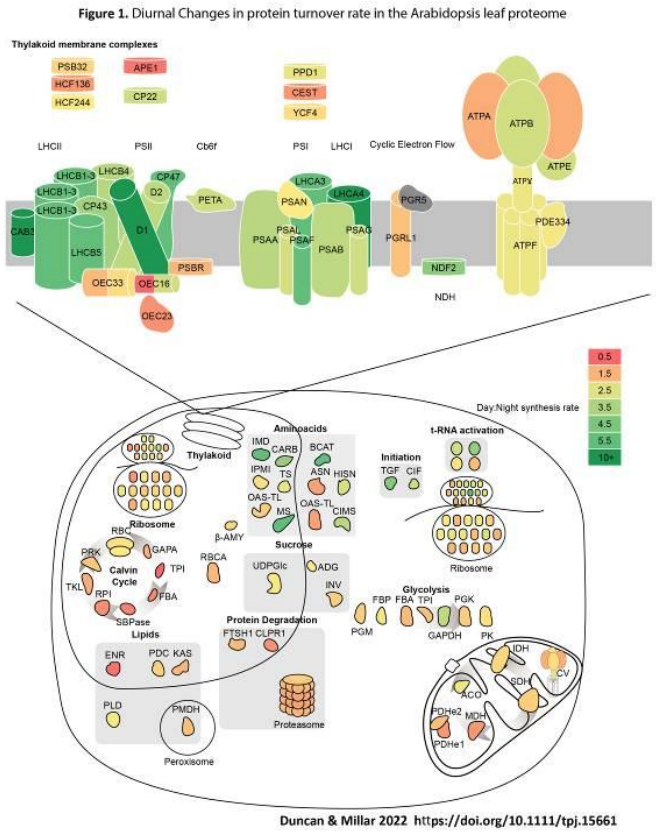
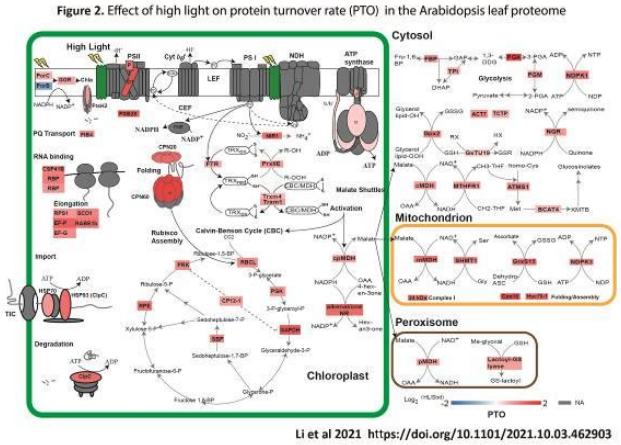


Fig. 2



IL12

Quantitative N-Terminomics of Protein TAILS in Protein Turnover Dynamics in COVID-19 and the SARS-3CLpro Substrate Landscape Identifies a Novel Intracellular Sesnor for Spike Glycoprotein

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Transcriptomics identifies mRNA levels, but not protein levels—yet whereas proteomics identifies protein levels, these do not necessarily reflect biological activity of the protein. Protein N and C terminal peptides provide critical information on protein function and stability. Our terminomics methods (TAILS, PICS) enrich and annotate terminomes, and our N and C-termini database TopFIND (https://topfind.clip.msl.ubc.ca) reveal widespread truncation and generation of termini in normal and diseased tissues. In the pandemic, we pivoted to understanding the pathogenesis of COVID-19 by identifying the SARS-CoV-2 3CLpro main protease human protein substrate repertoire. 3CLpro, the main viral protease, is indispensable for SARS-CoV-2 replication.

We delineate the interconnected human protein substrate landscape of 3CLpro using TAILS of human lung and kidney cells treated or not with interferons (N = 12), supported by analyses of SARS-CoV-2-infected lung cells. Over 100 substrate discoveries were identified and validated by MALDI-TOF analysis of synthetic peptide cleavage kinetics for each cleavage site in 100 substrates. Molecular docking simulations of 3CLpro engaging substrates confirmed the ~10% of sites validated were noncanonical that diverge from SARS-CoV-1 to guide substrate and inhibitor drug specificity. Cleaving the interactors of essential effector proteins, effectively stranding them from their binding partners, amplifies the consequences of proteolysis. Using recombinant protein digestion, Edman degradation, and digestion of normal human bronchial epithelial cell from 5 subjects further confirmed substrate cleavages. We show that 3CLpro targets multiple proteins in the Hippo pathway, including inactivation of MAP4K5, as well as key effectors of transcription, mRNA processing and translation. We demonstrate that Spike protein directly binds galectin-8, cleavage of which disengages CALCOCO2/NDP52 and decouples protective anti-viral-autophagy. Indeed, unlike healthy lung, in post-mortem COVID-19 human lung samples NDP-52 rarely colocalizes with galectin-8. The 3CLpro substrate degradome establishes a

foundational resource to accelerate further exploration of SARS-CoV-2 pathology in the COVID-19 *cellular coup d"etat*.

Pablos, I., et al and Overall, C.M. 2021. Mechanistic Insights into COVID-19 by Global Analysis of the SARS-CoV-2 3CLpro Substrate Degradome. Cell Reports 37, 1–17
DOI:https://doi.org/10.1016/j.celrep.2021.109892.

Fig. 1

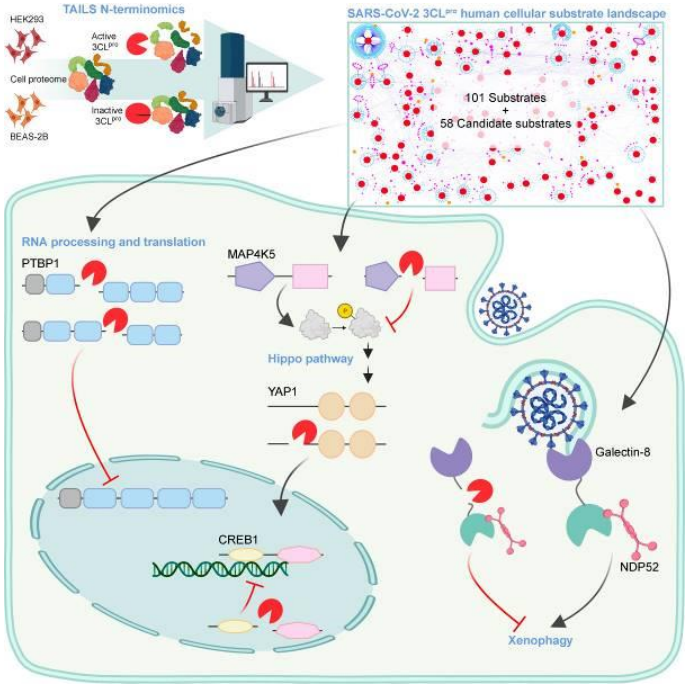
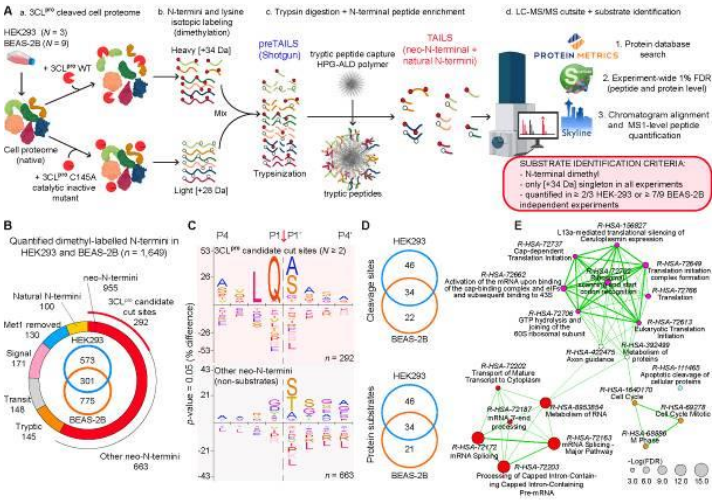


Fig. 2



IL13

***Brucella melitensis*: how proteomics could help to develop prophylactic measures**

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Brucellosis is a worldwide relevant zoonosis caused by Gram-negative bacteria belonging to the genus Brucella. To date, as much of eleven species of Brucella are known; of these, *Brucella melitensis* is among the most common in the Mediterranean areas causing brucellosis in humans and a variety of both small and large ruminants such as sheep, cows, camels and goats. Sheep and goats are the major reservoir of the pathogen, thus, representing the most important route for human infection through either the direct contact with the infected animals or indirectly by the consumption of contaminated animal-by products (e.g. raw milk). In the present study, we evaluate an alternative method for the fair identification of the immunogenic proteins specific of both B*. melitensis* Rev.1 and *B. melitensis* 16M. Specifically, our method aims at discriminating the *B. melitensis* antigens from those of the most cross-reactive specimens (i.e. *E. coli* O157:H7 and *Y. enterocolitica* O:9) providing knowledge on the exclusive immunogenic proteins of both the virulent and vaccinal strain. Sorting the major immunogenic proteins of both the vaccinal and field strain would be of pivotal importance for the design of unbiased serodiagnosis strategies to be employed in support to the conventional diagnostics for brucella. Also, it may serve as the starting point for drawing optimized prophylactic strategies targeting other molecules than those already addressed by the diagnostic tests. Amendment of diagnostic and prophylaxis in a DIVA perspective enables the fair discrimination of the infected from vaccinated animals, which currently represents one of the keystones for the adoption of fair and efficient eradication and

control plans against brucellosis in the humans and animals.

IL14

Critical Assessment of MetaProteome Investigation (CAMPI): a multi-laboratory comparison of established workflows

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[4]*Helmholtz-Centre for Environmental Research - UFZ GmbH, Leipzig, Germany*
[5]*Oak Ridge National Laboratory, Oak Ridge, TN, United States*
[6]*Université Paris Saclay, Bagnols-sur-Cèze, France*
[7]*Norwegian University of Life Sciences (NMBU), Ås, Norway*
[8]*Université Paris-Saclay, Jouy-en-Josas, France*
[9]*Robert Koch Institute, Berlin, Germany*
[10]*University of Minnesota, Minneapolis, MN, United States*
[11]*North Carolina State University, Raleigh, NC, United States*
[12]*Université Paris-Saclay, Gif-sur-Yvette, France*
[13]*University of Potsdam, Potsdam, Germany*
[14]*Bielefeld University, Bielefeld, Germany*
[15]*University of Sassari, Sassari, Italy*
[16]*Federal Institute for Materials Research and Testing, Berlin, Germany*

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 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. 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. 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.

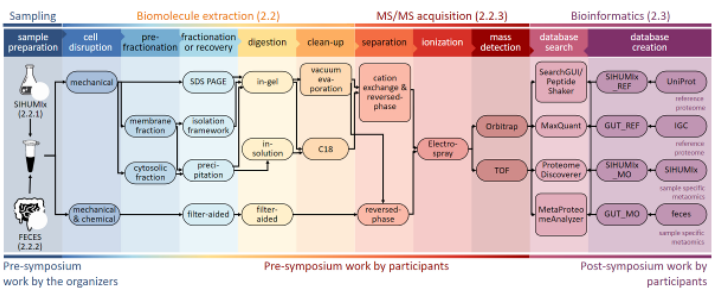
Results:

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.

Fig. 1



IL15

Clinical proteomics is easy – or not?

K. Barkovits[1]

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For disease, the goal of proteomics is to gain an understanding of disease mechanisms at the protein level by systematically profiling health and disease states as well as to discover new protein biomarkers for diagnostic purposes. However, we often face challenges which make it difficult to achieve this goal. These challenges start with the type of disease, the available sample type and its collection, and continue through sample processing and measurement to data analysis, which is finally subjected by technical and biological variances. Based on our analysis of cerebrospinal fluid (CSF), which is used as a source for biomarker research in the field of neurological diseases, different aspects of challenges are highlighted. In particular, emphasis is placed on the heterogeneity of clinical cohorts and the high variation in CSF protein composition and abundance between and within individuals. Based on our results, strategies for CSF biomarker studies will be outlined.

IL16

The metabolic and proteomic landscape of genome-scale genetic perturbation

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Metabolic reactions are vital for keeping cells and organisms growing and alive, and problems with cellular metabolism are implicated in ageing and diseases such as cancer, diabetes and brain disorders. Metabolism in the cell is organised in a genome-spanning network, known as the metabolic network, that connects several hundred enzymes with more than a thousand metabolites. In order to understand metabolism at its scale, novel technologies are required. These need to measure metabolites and proteins at precision, at high throughput, and at costs that facilitate systematic perturbation experiments at large scale. In this lecture, I''ll summarise our efforts in using mass spectrometry, yeast as a simple system, as well as human plasma analytics, for conducting hundreds to thousands of analytical measurements, allowing us to study how these complex metabolic processes are controlled/ Technical aspects of the lecture will include the summary of novel mass spectrometric acquisition techniques that centre around high-floware liquid chromatography to measure up to 1,800 samples per week per mass spectrometer, new acquisition schemes, Scanning SWATH, a new DIA-PASEF implementation and Zeno-SWATH, as well as new software that centres around the DIA-NN suite, developed in my laboratory. I'll further show unpublished results that i) demonstrate the acquisition of large numbers of plasma proteomes to study human metabolic disease, ii) a study that involved the acquisition of more than a 1,000 proteomes of yeast natural isolates to understand dosage compensation in genomic aneuploidies, and iii) the generation of a proteome for each non-essential yeast gene knock-out, to study the function of so far uncharacterized proteins.

IL17

Clinical (phospho)proteomics for precision medicine

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Clinical proteomics aims to disclose disease biology and drive improved diagnostics and treatment. I will highlight our work in various cancer and dementia, with a focus on biofluid and exosome proteomics for minimally-invasive diagnostics and phosphoproteomics for response prediction. I will discuss several biomarker/target discovery projects from wet lab strategies to data analysis in various disease applications. Altogether, we envision that clinical proteomics powered by precise measurements and dedicated analysis will realize the full potential of multi-parameter diagnostics and personalized medicine.

IL18

Tissue proteomics at single cell resolution

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The last decade has seen a renewed interest in developing mass spectrometry (MS)-based proteomics for clinical applications. This can increasingly take advantage of the rapid adances in the field of machine

learning and artificial intelligence. In this regard, I will introduce our open-source software suites called AlphaPept, which includes state of the art prediction of peptide properties from their sequence. Our group has developed a high sensitivity workflow for the analysis of pathology samples at the level of single cell types. The Bruker timsTOF has been improved, and in conjunction with a low flow adaption of the Evosep system, now allows analysis of single cells. This has revealed interesting properties of single cell transcriptomes vs. their proteomes. We have applied this technology for Deep Visual Proteomics (DVP), where we combine high resolution microscopy, automated image recognition by AI and ultrasensitive TIMS-PASEF analysis in data independent mode (diaPASEF). DVP merges powerful spatial imaging technologies with unbiased ultra-high sensitive proteomics to discern cell type-specific proteome change under normal and pathological conditions. We are currently applying this technology in oncology research and even in diagnosis of cancer patients.

IL19

The Spatial Organisation of the Cell

K. Lilley[1], E. Villanueva[1], T. Smith[1], M. Pizzinga[1], M. Elzek[1], R. Queiroz[1], R. Harvey[1], L. Breckels[1], O. Crook[2], M. Monti[1], V. Dezi[1], A. Willis[1]

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[2]*University of Oxford, Oxford, United Kingdom*

The complexity of living organisms does not scale with the predicted number of protein coding genes. Many factors contribute to increasing complexity, including non-coding RNA mediated control mechanisms and post-transcriptional and post-translation processing. The location of protein synthesis also plays a key role in expanding protein functionality, with aberrant spatial translation being a driver in multiple diseases.

I will describe our approaches to capture the spatial relationship between RNA and protein on a cell-wide scale. I will discuss methods designed to map the cellular spatial proteome on a cell-wide scale(1) (2) based on physicochemical fractionation of cellular components (LOPIT), give examples of how we have applied these methods to interrogate many different biological scenarios (3) (4) (5) and describe associated robust computational workflows (6).

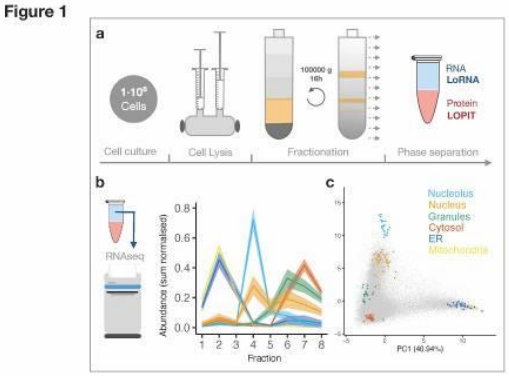
The main emphasis of my talk, however will focus on the development of a new method that allows the simultaneous mapping of the both the spatial proteome and spatial transcriptome on a cell-wide scale. Using the same set of samples, we combine LOPIT with a new approach, LoRNA (the localisation of RNA) (7).

LoRNA–derived data has allowed us to determine a larger than assumed cohort of lncRNAs located in the cytosol, and to develop rules about which RNA features control subcellular partitioning.

Using this approach we have mapped the concerted relocalisation of the transcriptome and proteome upon the Unfolded Protein Response (UPR). Our data give insights into proteins and RNA species that co-locate to stress granules upon UPR. We show a sub-set of mRNA species that persist at the Endoplasmic Reticulum during this response.

1. Mulvey et al (2017) Nature Protocols - doi: 10.1038/nprot.2017.026
2. Geladaki et al (2019) Nature Commun. - doi:10.1038/s41467-018-08191-w
3. Barylyuk et al (2020) Cell Host & Microbe - doi: 10.1016/j.chom.2020.09.011
4. Shin et al (2020) Nature Commun. - doi: 10.1038/s41467-020-19840-4
5. Mulvey et al (2021) Nature Comm - doi: 10.1038/s41467-021-26000-9
6. Crook et al (2022) in revision and bioRxiv 2021.01.04.425239

Fig. 1



IL20

Interactomics by co-fractionation-mass spectrometry: evolving informatics and applications

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The ability of proteins to interact to form quaternary and higher-order complexes underlies all of biology. Co-fractionation mass spectrometry (CF-MS) has emerged as a powerful, scalable technique for mapping these interactions. As with any area of science, the methods (both wet-bench and informatic) behind this continue to evolve. There is little consensus on optimal strategies for the design of CF-MS experiments or their analysis. I will discuss our recent efforts to further optimze the whole experimental workflow, from sample preparation to biological interpretation. This will include a meta-analysis of all published data to derive optimal conditions, as well as efforts to move away from "gold-standard" reference interactions, yet still remain unbiased by existing data. Lastly, I will discuss our efforts to extend these approaches to non-model organisms where a paucity of functional molecular knowledge about the proteins present makes interpretation extremely challenging

IL21

Phenotyping cellular states with biophysical proteomics

M. Savitski[1]

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Thermal stability of proteins can be measured in living cells on a proteome-wide scale using thermal proteome profiling, TPP. While the technology was initially developed to identify drug targets, the improvements in sensitivity now make it possible to detect thermal stability changes which result from modulation of protein-protein, protein-metabolite, protein-DNA interactions etc. Thus TPP has the ability to phenotype cellular states and capture functionally relevant changes not accessible to expression proteomics. Recently we combined TPP with reverse genetics to map the functional landscape of the model organism E. coli. This study and further unpublished work will be presented in the talk.

IL22

Multimodal Targeted Glycoproteomics and Glycan Imaging Mass Spectrometry of FFPE Tumor Tissues

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Question: N-glycan imaging mass spectrometry (IMS) analysis of formalin-fixed human tumor tissues has been highly effective at identifying N-glycan distributions associated with distinct tumor, stroma and immune regions. The N-glycans are released by spraying a molecular coating of peptide N-glycosidase, and the resulting molecular maps can be used to identify regions of interest for further glycoproteomic analysis.

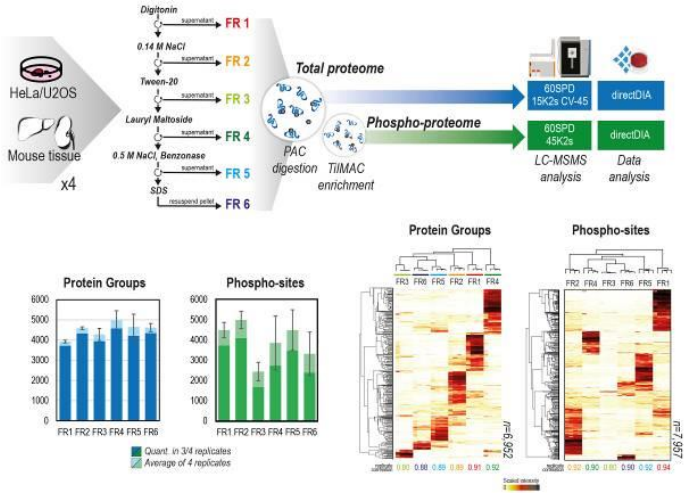
Methods: Multiple analysis workflows were performed on human formalin-fixed prostate, pancreas and colon cancer tissues. These included immunohistochemistry (IHC) of known carbohydrate tumor antigens and glycoproteins, different proteases, and bioorthogonal reagents for sialic acids. All approaches were linked to the N-glycan IMS tissue maps, generated on a Bruker MALDI-QTOF timsTOF fleX instrument. Images were visualized with SCiLS Lab software.

Results: IHC analysis of carbohydrate tumor antigens like sialyl Lewis A/CA19-9, sialyl Lewis X and fucosylated Lewis Y structures in tissues was combined with N-glycan IMS for analysis of pathologist-annotated tumor tissues. A series of formalin-fixed paraffin embedded tumor tissues from prostate, colon, and pancreas were processed for standard IHC analysis. Each slide was scanned by high resolution microscopy, followed by removal of the coverslip, an antigen retrieval heating step, and PNGase F digestion. The IHC-stained slide is used as the template for teaching coordinates and is already positioned for co-localizing any N-glycans detected by MALDI IMS. In tumor regions, the majority of N-glycans co-localizing with Lewis Y staining were branched tetraantennary structures with 2-9 fucose residues. For sialyl Lewis A or X antigen co-localization, the detected glycans were multi-sialylated and multi-fucosylated branched structures. The workflow is adaptable to use with other IHC targets like common diagnostic tumor antigens, glycoproteins and glycosyltransferases. Further, alpha-2,3 linked sialic acid N-glycans that comprise the sialyl Lewis A/X motif can be specifically targeted. Prior to PNGase F digestion, the tissues can be treated chemically with an amidation reaction for alpha 2,6 isomers, followed by use of a amine-azide that

specifically targets alpha-2,3 linked sialic acids. The N-glycan and IHC stained images can be used to scrape off regions of interest directly from the tissue, and the lysate used for chemical affinity enrichment of the alpha-2,3 sialylated glycoproteins. Biotin-alkyne in solution or attached to beads is used for click chemistry conjugation. Different proteases can be added for digestion off the beads and peptide identification by tandem MS. Alternatively, streptavidin fluor can be used to visualize the single cell distributions of the sialic acids in intact tissues.

Conclusions: Overall, the approach provides an integrated workflow to efficiently link tumor antigen pathology assessment with imaging MS and glycoproteomic MS workflows.

Fig. 1



IL23

Phosphoproteomics of cancer signaling

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[2]*University, Copenhagen, Germany*

Here we describe a new spatial phosphoproteomics technology that is based on a simple chemical fraction method for high-throughput and reproducible analysis of subcellular phosphoproteomes by using short LC gradients and data-independent acquisition (DIA). The subcellular analysis workflow is based on sequential cell fractionation to profile the global proteome and phosphoproteome dynamics across six distinct subcellular fractions. We have applied the workflow to study spatio-temporal EGFR phospho-signaling dynamics in-vitro in HeLa cervix carcinoma cells and in-vivo in mouse tissues. We have also investigated the spatio-temporal stress signaling induced by osmotic shock in U20S osteosarcoma cells revealing cellular relocation of ribosomal proteins in response to hypertonicity and in muscle contraction. Finally, we have used the workflow to study the impact of a cancer therapeutic drug on the subcellular (phospho)proteome in sensitive and resistant acute myeloid leukemia (AML) cells. Our spatial proteomics method is a powerful strategy for studying phospho-signaling dynamics at subcellular resolution.

IL24

Proteogenomics to connect cancer genotype with molecular phenotype for improved treatment selection

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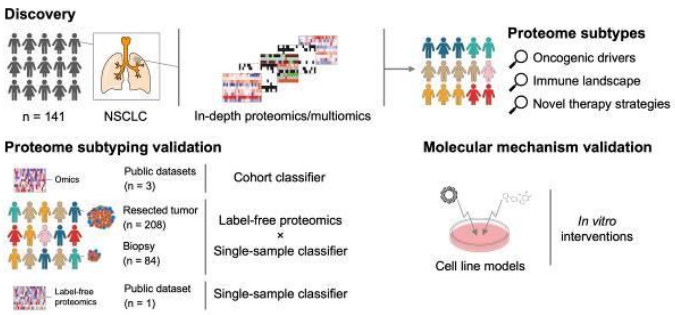
The explosion of genomics data has improved our understanding of cancer greatly in recent years. However, the systems level knowledge on how genomic aberrations affect the functional proteome is still very limited, hindering effective selection of anti-cancer drug combinations in precision medicine. Proteome data represents the combined effect of epigenetic, transcriptional and translational regulation and will therefore provide an important molecular phenotype data layer for multi-omics analysis. To allow effective systems biology analysis including proteomics, we have generated tools that take advantage of massive genomics and transcriptomics data by incorporating sequence information to the proteomics data-analysis pipeline. This will allow protein level analysis of gene variants as well as detection of novel protein coding regions. In cancer, genomics instability creates so called neoantigen when non-canonical proteins and peptides are translated. Our proteogenomics methods offers a view into tumor neoantigen space by controlling error rate in variant peptide detection by combining experimental isoelectric point data from peptide fractions (HiRIEF LC-MS/MS) and bioinformatics approaches into the proteogenomics workflow (IPAW). In same analysis, in-depth quantitative proteome data on tumor tissue

provides information both on cancer driving pathways and molecular insights in immune evasion mechanisms.

Here we demonstrate the value of proteogenomics driven multi-omics view of cancer by analysis of several lung cancer cohorts. We could define proteome-based lung cancer subtypes using HiRIEF LC-MS based in-depth proteomics of 141 cases. Interestingly, these subtypes differ on tumor neoantigen burden and types of neoantigens and this was related to immune evasion mechanisms. A lung adenocarsinoma subtype associated with poor prognosis, immune-cold phenotype, despite high neoantigen burden, was identified. This subtype was enriched in STK11 mutations and we could demonstrate that this activated immune evasion mechanism via expression of FLG1 immune inhibitory ligand.

To allow clinical application, we developed a DIA based classifier for lung cancer subtyping. The classifier was tested in two additional tumor cohort, namely in a similar early-stage lung cancer cohort of 208 cases and using a late-stage biopsy cohort of 80 samples.

Fig. 1



IL25

The advent of epi-proteomics: Our first steps into an exciting new world!

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Proteomics advances have provided ever more analytical depth, progress that has bene powered by novel approaches, by more performant mass spectrometers, and by ever more sophisticated data analysis algorithms. But we know there is more to proteins than their primary sequence. Indeed, the function of most proteins is linked to their localisation, and with the proteins they interact with. Nevertheless, despite this long-standing insight, we still know remarkably little about how proteins are controlled and regulated in living cells, or how these mechanisms differ between different cells. Of course, well-known modifications such as protein phosphorylation are quite well documented, but many other protein modifications remain largely unexplored. As a result, interesting downstream phenomena, such as competition between, or coordination of, modifications on the same site or the same protein remain underexplored.

Interestingly, a wholly new generation of machine learning-based identification tools is now radically changing our ability to discover the proteome-wide modification landscape, with ionbot (https://ionbot.cloud) a forerunner among these revolutionary new tools. ionbot is based on the MS^{2}PIP (https://iomics.ugent.be/ms2pip) and DeepLC (http://compomics.github.io/projects/DeepLC) models to predict analyte behaviour, is fast and reliable, and allows unbiased identification of protein modifications at unprecedented scale. Moreover, we have shown that the use of machine learning models can dramatically enhance various other challenging areas of proteomics analysis, including immunopeptidomics, .

To show the capabilities of ionbot, and to cast a first glance at the true complexity of a proteome as our experiments and instruments have seen it, we have applied ionbot to over 1 billion human spectra, and over 600 million mouse spectra from the PRIDE archive (https://www.ebi.ac.uk/pride), uncovering a plethora of modifications of various origin and level of interest. This wholly new view of the modified proteome dramatically changes our view on proteins, and shows the overwhelming abundance of chemical, biological, or artefactual modifications that affect the protein machinery of life.

In the past, we already leveraged such data to infer protein associations (https://iomics.ugent.be/tabloidproteome), but recent advances in machine learning have allowed us to dramatically expand our coverage, in turn yielding a massive increase in the (less) biased detection of protein association or interactions from high-throughput omics data.

We can also use these proteome-wide results to analyse tissues and even cell types for their protein

composition, yielding detailed information on tissue-specific proteomes with potentially far-reaching applications.

IL26

Reactome Integrative Pathway Analysis

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Reactome (https://reactome.org) is a free, open-source, open-data, curated and peer-reviewed knowledge base of biomolecular pathways, currently covering 11,270 proteins in 13,960 reactions supported by 34,252 literature references. Pathways are arranged in a hierarchical structure, allowing the user to navigate from high level concepts like immune system to detailed pathway diagrams showing biomolecular events like membrane transport or phosphorylation.

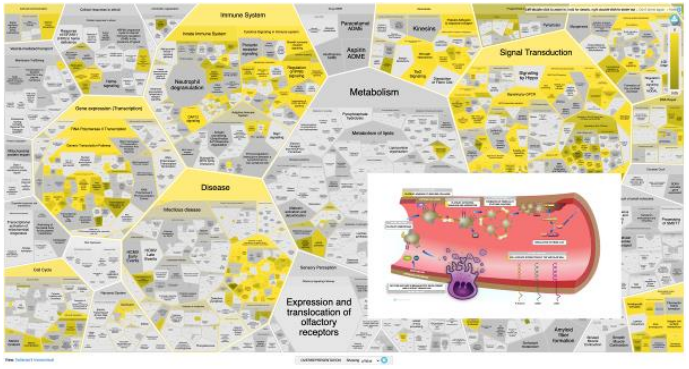
For the higher levels of the hierarchy, Reactome provides scalable, interactive textbook-style diagrams in SVG format, which are also freely downloadable and editable. Repeated diagram elements like 'mitochondrion' or 'receptor' are freely available as a library of graphic elements at https://reactome.org/icon-lib. Detailed lower-level diagrams are downloadable in editable PPTX format as sets of interconnected objects, as well as in standard png format.

Pathway analysis capabilities range from simple gene enrichment to quantitative multi-omics analysis with ReactomeGSA. Higher confidence interactors from the IntAct database can be integrated in the analysis. Analysis can be submitted through an R interface, and results are presented interactively in a visually attractive genome-wide results overview based on Voronoi maps. Downloads are available in textual as well as graphical formats.

In addition to molecular interactions from external databases, known disease associations from DisGeNET can now be overlayed on the pathway visualisations.

Reactome follows an open source, open data policy, all data and source code are available under permissive licences.

Fig. 1



IL27

Kinase signalling circuits in health and disease

P. Beltrao[1]

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Cells need to constantly adapt to changes in conditions and use post-translational regulation as a fast way to transfer information from sensors to effectors of cellular responses. Advances in mass-spectrometry allow us to identify post-translational modification (PTMs) sites on a large scale and to quantify their changes across different conditions. However, the interpretation of these measured changes remains challenging. We have worked on approaches that try to predict the kinase-kinase regulatory network and how to use large scale phosphoproteomics to infer the activation state of kinases. As an example we have applied these approaches to study the changes in kinase signalling across tumour samples or occurring during SARS-CoV-2 infection. Our work on the tumour samples revealed a disconnect between the mutational status of the tumour and the predicted activation state of kinases, indicating substantial compensatory mechanisms. Based on the viral phosphorylation studies we could show how SARS-CoV-2 infection promoted casein kinase II (CK2) and p38 MAPK activation and the inhibition of cell-cycle kinases. These were linked to production of diverse cytokines, cell cycle arrest and stimulation of filopodial protrusions. These approaches are starting to give us a less biased understanding of the kinase signalling network and uncovering the importance of phospho-regulation across multiple aspects of cell biology and disease.

IL28

Functional characterisation of human phosphorylation sites

M. Selbach[1]

[1]*Max Delbruck Center for Molecular Medicine, Berlin, Germany*

Phosphoproteomics routinely quantifies changes in the levels of thousands of phosphorylation sites, but functional analysis of such data remains a major challenge. I will present three different ways to characterise the function of human phosphorylation sites. First, I will show how data from in vitro kinase assays can be used to predict kinase activity in phosphoproteomic datasets. Second, I will show quantitative affinity purification experiments with synthetic phosphopeptides can help to asses their cellular function. Finally, I will outline how quantitative RNA-interactome capture (qRIC) can quantify the fraction of cellular RNA-binding proteins that are pulled down with polyadenylated mRNAs. Combining qRIC with phosphoproteomics allows us to systematically compare pull-down efficiencies of phosphorylated and non-phosphorylated forms of RBPs. Using qRIC, we identify over hundred phosphorylation sites with regulatory potential, including known regulatory sites. Follow-up experiments on the cardiac splicing regulator RBM20 revealed that multiple phosphorylation sites in the C-terminal disordered region affect nucleo-cytoplasmic localisation, association with cytosolic RNA granules and alternative splicing.

IL29

Light-mediated discovery of surfaceome nanoscale organization and inter-cellular receptor interaction networks

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The molecular nanoscale organization of the surfaceome is a fundamental regulator of cellular signaling in health and disease. Technologies for mapping the spatial relationships of cell surface receptors and their extracellular signaling synapses would unlock theranostic opportunities to target protein communities and the possibility to engineer extracellular signaling. Here, we develop an optoproteomic technology termed LUX-MS that enables the targeted elucidation of acute protein interactions on and in between living cells using light-controlled singlet oxygen generators (SOG). By using SOG-coupled antibodies, small molecule drugs, biologics and intact viral particles, we demonstrate the ability of LUX-MS to decode ligand receptor interactions across organisms and to discover surfaceome receptor nanoscale organization with direct implications for drug action. Furthermore, by coupling SOG to antigens we achieved light-controlled molecular mapping of intercellular signaling within functional immune synapses between antigen-presenting cells and CD8+ T cells providing insights into T cell activation with spatiotemporal specificity. LUX-MS based decoding of surfaceome signaling architectures thereby provides a molecular framework for the rational development of theranostic strategies.

IL30

Defining mitochondrial protein functions through deep multi-omic profiling

D. Pagliarini[1]

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Mitochondria are epicenters of eukaryotic metabolism and bioenergetics. Pioneering efforts in recent decades have established the core protein componentry of these organelles and have linked their dysfunction to over 150 distinct disorders. Still, hundreds of mitochondrial proteins lack clear functions, and ~40% of mitochondrial disorders remain unresolved. To establish a more complete functional compendium of human mitochondrial proteins, we profiled more than 200 CRISPR-mediated HAP1 cell knockout lines using mass spectrometry-based multi-omics analyses. This effort generated ~8.3 million distinct biomolecule measurements, providing a deep survey of the cellular responses to mitochondrial perturbations, and laying a foundation for mechanistic investigations into protein function. Guided by these data, we discovered that PYURF is a SAM-dependent methyltransferase chaperone that supports both complex I assembly and coenzyme Q biosynthesis, and that is disrupted in a previously unresolved multisystemic mitochondrial disorder. We further linked the putative zinc transporter SLC30A9 to mitoribosome and OxPhos integrity and established *RAB5IF* as the second gene harboring pathogenic variants causing cerebrofaciothoracic dysplasia. Our data—which can be explored through an interactive online MITOMICS.app resource—suggest biological roles for

many other orphan mitochondrial proteins still lacking robust functional characterization, and define a rich cell signature of mitochondrial dysfunction that can support the genetic diagnosis of mitochondrial diseases.

Citation:

Defining mitochondrial protein functions through deep multi-omic profiling

Rensvold JW*, Shishkova E*, Miller IJ, Sverchkov Y, Cetinkaya A, Pyle A, Manicki M, Brademan DR, Alanay Y, Raiman J, Jochem J, Peters SR, Linke V, Hutchins PD, Overmyer KA, Salome AZ, Hebert AS, Balnis CV, Kwiecien NW, Rush MJ, Westphall MS, Craven M, Akarsu NA, Taylor RW, Coon JJ†, and **Pagliarini DJ†**

Nature, Accepted manuscript

IL31

Development of a Novel Single-Molecule Proteomics Analysis Platform based upon Protein Identification by Short epitope Mapping

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The proteome is perhaps the most dynamic and valuable source of functional biological insight. Here, we introduce a framework for a novel affinity-reagent-based single-molecule protein analysis approach that includes novel biochemistry for single-protein molecule immobilization, instrumentation for highly sensitive multi-cycle probing of those protein molecules, and machine learning for data interpretation. We call the approach Protein Identification by Short-epitope Mapping (PrISM). PrISM utilizes multi-affinity reagents to target short linear epitopes with both a high affinity and low specificity. PrISM further employs a novel protein decoding algorithm that considers the stochasticity expected for single-molecule binding. In simulations, PrISM is able to identify more than 98% of proteins across the proteomes of a wide range of organisms. PrISM is robust to potential experimental confounders including false negative detection events and noise. Simulations of the approach with a chip containing 10 billion protein molecules show a dynamic range of 11.5 and 9.5 orders of magnitude for blood plasma and HeLa cells, respectively.

IL32

Imaging the proteome in context: Innovation in translational technologies

R. Heeren[1]

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Modern molecular analytical technologies in the "omics" arena play a crucial role in many scientific disciplines ranging from material sciences to clinical diagnostics. Molecular pathology is no different. Technological advances have increased methodological sensitivity allowing researchers to acquire molecular information of smaller and smaller samples. The biggest challenge is to put that concerted 'omics' information in the context of the problem the samples originate from. This lecture describes how innovative molecular imaging technologies, based on mass spectrometry and "omics" innovations have impacted translational clinical research. Innovative imaging technologies now offer new insights in life"s complexity that can be employed for precision medicine, the understanding of new (bio)materials and the processes that happen on the interface of living and "dead" matter. Innovations in mass spectrometry based chemical microscopes have now firmly established themselves in translational molecular research. One key aspect of translational success is the ability to obtain this molecular information on thousands of molecules on a process relevant timescale and at the single cell level. Modern mass microscopes can now rapidly acquire images of metabolites, lipids, polymers, peptides and proteins, depending on the spatial resolution chosen. Combined they offer a truly precision molecular imaging approach that reveals molecular complexity in the context of its biological and material environment.

IL33

Antigen discovery for development of personalized cancer immunotherapy

M. Bassani-Sternberg[1]

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The remarkable clinical efficacy of the immune checkpoint blockade therapies has motivated researchers to discover immunogenic epitopes and exploit them for personalized vaccines and T cell based therapies. Mutated human leukocyte antigen binding

peptides (HLAp) are currently the leading targets. We and others have shown that the direct identification of immunogenic neoantigens by mass spectrometry (MS) is feasible. In contrast to mutated neoantigens that are often patient-specific, tumor-specific antigens that are shared across patients may be more attractive for immunotherapy. Recent studies have focused on the discovery of aberrantly-expressed non-canonical antigens, which expand the repertoire of targetable epitopes through the translation and presentation of presumably non-coding regions. We have developed a dedicated computational pipeline called NeoDisc that can precisely characterize the antigenic landscape of tumors, incorporating whole exome sequencing, transcriptomics, MS-based immunopeptidomics and advanced HLA binding prediction tools. NeoDisc can lead to the direct (MS-based) identification of mutated neoantigens by MS as well as non-canonical cancer–specific peptides derived from unconventional coding sequences in the genome, and it predicts and prioritize the most likely immunogenic antigens. Since 2020, we apply this antigen discovery approach in cancer vaccine and adoptive transfer of neoantigen-specific T cells phase I clinical trials. This platform holds great promise for the discovery of novel and actionable cancer antigens for cancer immunotherapy.

IL34

Dynamic organelle structure-function relationships during viral infections

I. M. Cristea[1]

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Organelle remodeling is an essential component of all virus infections. Changes in organelle composition, shape, and function underlie mechanisms that either support virus replication or inhibit host defense responses during infections. Here, we investigate functional organelle remodeling events induced by both ancient and rapidly evolving human viruses: human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1), influenza A (Infl. A), and the beta-coronavirus HCoV-OC43. We integrate quantitative proteomics with live cell and super-resolution microscopy, virus-host protein interaction studies, and metabolomics assays. Using this interdisciplinary approach, we go on to address the long-standing question of how HCMV, despite triggering mitochondria fragmentation, succeeds to upregulate mitochondrial bioenergetics to support virus production. We identify a previously uncharacterized viral protein that alters the mitochondrial ultrastructure and increases oxidative phosphorylation. We further investigate the balance between antiviral and proviral processes within the mitochondria. We place these findings within our ongoing efforts to globally characterize organelle remodeling events and to define at a systems-level the temporal rewiring of protein-protein interactions during the progression of viral infections. Altogether, these studies offer molecular fingerprints of organelle remodeling linked to infection and pathogenesis.

IL35

Mass Spectrometry to detect Sars-Cov-2 in infectious patients: amplifying the signal-to-noise, not the target molecule

M. Dhaenens[1]

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The pandemic readiness toolbox needs to be extended, providing diagnostic tools that target different biomolecules, using orthogonal experimental setups and fit-for-purpose specification of detection. We have built on a previous Cov-MS effort that used liquid chromatography-mass spectrometry (LC-MS) and present a method that allows accurate, high throughput measurement of SARS-CoV-2 nucleocapsid (N) protein.

We used Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) technology to enrich and quantify proteotypic peptides of the N protein from trypsin-digested samples from COVID-19 patients. The Cov²MS assay was shown to be compatible with a variety of sample matrices including nasopharyngeal swabs, saliva and blood plasma and increased the sensitivity into the attomole range, up to a 1000-fold increase compared to direct detection in matrix. In addition, a strong positive correlation was observed between the SISCAPA antigen assay and qPCR detection beyond a quantification cycle (Cq) of 30-31, the level where no live virus can be cultured from patients. The automatable "addition only" sample preparation, digestion protocol, peptide enrichment and subsequent reduced dependency upon LC allow analysis of up to 500 samples per day per MS instrument. Additionally, we show that the test is insensitive to patient sample pooling and that variants

of concern as well as influenza A and B and RSV are also detectable in a readily multiplexed assay.

In doing so, we provide the future perspective of a diagnostic niche not directly competing with qPCR. More specifically, MS can develop into an early warning system that targets several (respiratory) pathogens in pools of up to 32 patient samples. With each MS instrument being able to run 500 pooled samples a day, over 15,000 individuals can soon be monitored for a dozen circulating pathogens in the population every day on a single instrument, providing true pandemic preparedness capabilities.

IL36

The SARS-CoV-2 RNA-protein interactome at subgenome resolution

N. Schmidt[1], Y. Wei[1], S. Ganskih[1], C. A. Lareau[2], H. Keshishian[3], S. Zielinski[1], J. Vogel[1,4], J. Bodem[4], S. A. Carr[3], ***M. Munschauer***[1,4]

[1]Helmholtz-Institute for RNA-based Infection Research, Wuerzburg, Germany

[2]School of Medicine, Stanford University, Palo Alto, CA, United States

[3]Broad Institute of MIT and Harvard, Cambridge, MA, United States

[4]University of Wuerzburg, Wuerzburg, Germany

Characterizing the interactions that SARS-CoV-2 viral RNAs make with host cell proteins during infection can improve our understanding of viral RNA functions and the host innate immune response. Using RNA antisense purification and mass spectrometry (RAP-MS), we have recently characterized the first atlas of human and viral proteins that directly and specifically bind to SARS-CoV-2 RNAs in infected human cells. We now expand these results to different cell types and resolve the interactomes of genomic and subgenomic viral RNAs separately. We map direct interaction sites of viral and host cell proteins on SARS-CoV-2 RNAs and use genetic perturbation together with pharmacological inhibition to demonstrate the functional relevance of several direct RNA binders in SARS-CoV-2 infections. The identification of host dependency factors and defense strategies as presented in this work provides a general roadmap for dissecting the biology of RNA viruses and the interactions between hosts and pathogens at the molecular level with therapeutic implications.

IL37

Translational Potential of Population Proteomics

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Measuring thousands of proteins circulating in blood is now possible at population-scale through the development of high-throughput affinity assays. Clinical actionable insights, however, have been limited so far due to the unclear origin of the vast majority of proteins measurable from blood samples and the ubiquitous sources of unknown confounding in observational studies. In this talk I will show how causal relationships between proteins and diseases with potential for therapeutic applications can be established when anchored on a shared genetic architecture. Specifically, I will introduce genome-proteome-wide association studies involving >10k participants to detect human DNA sequence variants strongly associated with variations in plasma protein levels, so-called protein quantitative trait loci (pQTL), and how our team used those to chart a map of the proteogenomic convergence of human health. Apart from novel specific protein-disease relationships, the map revealed examples in which a protein is putatively causally linked to multiple outcomes across clinical domains, such as Fibulin-3 being linked >30 outcomes, including diverse connective tissue disorders resembling mouse knock-out models. I will further demonstrate that half of the protein targets for which we identified a pQTL close to the protein-encoding gene are linked to gene expression events in selective or multiple tissues providing unique insights how diverse tissues contribute to the composition of the blood proteome.

IL38

Monitoring Health and COVID-19 Using Big Data

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Recent technological advances as well as longitudinal monitoring not only have the potential to improve the treatment of disease (Precision Medicine) but also empower people to stay healthy (Precision Health). We have been using advanced multiomics technologies (genomics, immunomics, transcriptomics, proteomics, metabolomics, microbiomics) as well as wearables for monitoring health in 109 individuals for up to 12 years

and made numerous major health discoveries covering cardiovascular disease, oncology, metabolic health and infectious disease. We have found that individuals have distinct aging patterns that can be measured in an actionable period of time as well as seasonal patterns of health markers. We have also explored the effects of fiber using multiomics profiling. Finally, we have used wearable devices for early detection of infectious disease, including COVID-19 and built an alerting system for detecting health stressors that is scaleable to the entire planet. We believe that advanced technologies have the potential to transform healthcare.

IL39

Two Sides of Precision Medicine: Individualizing Assessment and Therapies

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Underlying precision medicine is the concept that an individual"s Omic (proteomic) signature will provide a physician with clinically actionable diagnosis and a subsequent mechanistic therapeutic route. The challenge remains in the identification of treatable diagnosis for each specific individual. To accomplish this will require i) having an array of mechanistic therapies for each disease and ii) a means to diagnosis (identify) which therapy (or combination) will be appropriate for a particular person. We have developed a multiple modal (plasma and dried blood) high throughput LC-MSMS proteomic pipeline which we have deploy to track individuals with COVID-19 as well as patients with mid-risk cardiac diseases. Simultaneously, we are developing scalable methods for high-through methods for mechanistic drug screening on iPSC-derived cultured tissue or single cell isolated from tissue biopsies from target individuals who could be beneficial in the identification of new and or the assignment of the appropriate drug treatment. Focusing first on cardiomyopathies including COVID-19 long haulers. Only by addressing both individual"s assessment and development of individual mechanistic treatment will precision medicine be successful.

IL40

On the Uniqueness of our Plasma Proteome and Immunoglobulome

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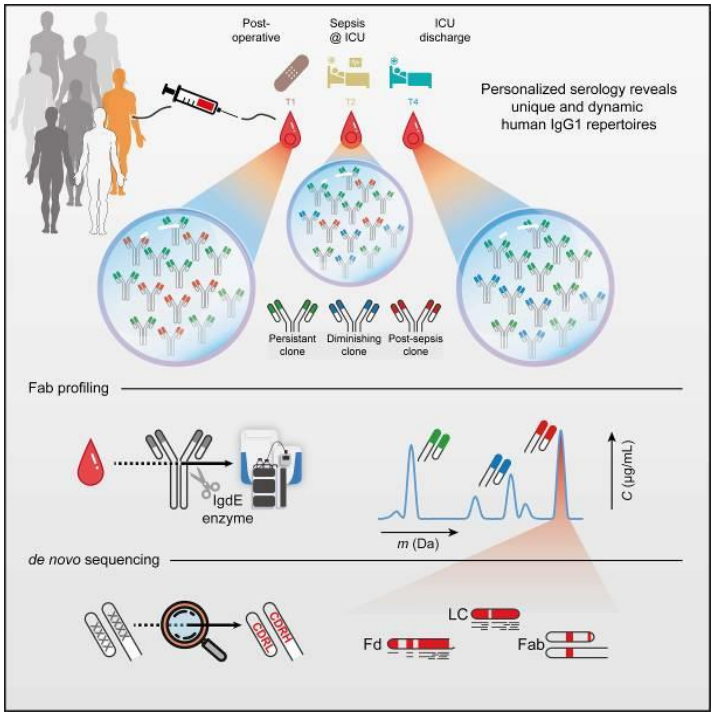
High-resolution (native) mass spectrometry enables the mass analysis of intact proteins and protein assemblies, ranging from antibodies to naïve viruses with molecular weights beyond 10 MDa. In this lecture some recent advances in this field will be highlighted, including extending the attainable mass range, the sensitivity of detection, the mass resolving power and the proteoform specificity. Cumulatively, these advances have initiated new applications, not only in the detailed characterization of advanced biopharmaceuticals, but also in analysis of the plasma (glyco)proteome.

Serum glycoproteins are characterized by a plethora of proteoforms, due to glycosylation and phosphorylation. High-resolution native MS can be used for comprehensive proteoform profiling. Unlike most clinical assays, this approach enables the monitoring of not only protein abundance, but also proteoform abundance, allowing us to address an intriguing question; How unique and personalized is our serum (glyco)proteome?

In the second part I will focus on the direct analysis of serum immunoglobulins. The human body produces immunoglobulins (Igs) to recognize and combat pathogens. It has been estimated that our bodies can theoretically produce several billions of distinct IgG1 variants. Seemingly in contradiction, we observe that circulating IgG1 repertoires are dominated by only a few dozens of clones. We demonstrate that 1) personalized IgG1 profiling by LC-MS is feasible, 2) each person exhibits a unique serological IgG1 repertoire, 3) this repertoire adapts to changes in physiology, and 4) that individual plasma IgG clones can be *de novo* sequenced by integrative protein-centric and peptide-centric proteomic approaches. We foresee that the presented approach will accommodate more rapid development of monoclonal antibody treatments, immediately assessing fully human, matured, and optimized molecules.

Fig. 1



IL42

Dynamic Organellar Maps: A versatile tool for phenotype discovery

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We have harnessed the power of spatial proteomics to uncover the pathomechanisms of AP-4-deficiency syndrome, a severe neurological disease. AP-4 is a complex required for sorting proteins into transport vesicles at the trans-Golgi network. We applied our Dynamic Organellar Maps (DOMs) approach in conjunction with quantitative imaging and lipidomics to identify functions of the AP-4 pathway. This revealed that AP-4 mediates axonal targeting of ATG9A, a protein required for autophagy, and the lipase DAGLB, a key enzyme for endocannabinoid production. Lack of AP-4 thus damages neurons in two ways: First, through dysregulated axonal autophagy, leading to neurodegeneration; and second, through a reduction of lipid signalling, which restricts axonal growth during neuronal development. Based on this disease mechanism model, we propose that pharmacological modulation of endocannabinoid metabolism may be a therapeutic strategy for treating AP-4 deficiency. Furthermore, we have recently incorporated data-independent acquisition of MS data into the Dynamic Organellar Maps workflow, which drastically enhances the performance. Relative to our standard workflow, DIA-DOMs achieve twice the mapping depth per MS run time. We have applied DIA-DOMs to capture subcellular localization changes induced by starvation/disruption of lysosomal pH, revealing a subset of Golgi proteins that cycle through endosomes. These two studies highlight the power of DOMs for unbiased and systematic phenotype discovery.

IL43

Peptide-level mass spectrometry imaging of solid tumors using reproducible data analysis workflows

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Mass spectrometry imaging (MSI) of tryptic peptides is frequently applied for protein imaging directly from formalin-fixed and paraffin-embedded (FFPE) tissue slices archived at pathologies. MSI allows studying solid tumors in a spatially resolved manner and thus gives new insights into tumor biology by measuring intra-

tumor heterogeneity and the spatial composition of the tumor microenvironment. Furthermore, it identifies spatio-molecular signatures that stratify tissues according to different diagnoses or prognoses. The data generated by MSI is typically large and complex and requires specialized software for data analysis. For peptide MSI experiments, typically interactive proprietary software is used, which hinders standardized and reproducible data analysis.

We have previously built a comprehensive suite of MSI data analysis tools in the open source bioinformatic platform Galaxy (https://usegalaxy.eu). The tools are built in a modular way and can be assembled into standardized Galaxy workflows. Workflows as well as complete analysis histories can be shared in Galaxy and meet the criteria of FAIR (findability, accessibility, interoperability, and reusability) research data. We have used Galaxy to analyze tryptic peptide MALDI-TOF imaging datasets of 39 urothelial carcinoma, >50 pancreatic cancers, and whole-slide ion images or matched colorectal primary tumor -liver metastasis.

In the urothelial carcinoma dataset, we classified tumor and surrounding stroma tissues as well as muscle-infiltrating and non-muscle infiltrating urothelial cancers with very high classification accuracy. Immunohistochemistry confirmed an increased histone H2A abundance in the tumor compared to the stroma tissues and increased vimentin and decreased cytokeratin 7 in muscle-infiltrating vs. non-muscle infiltrating urothelial carcinomas. In the pancreatic cancer experiment, two tumor subtypes, pancreatic ductal adenocarcinoma and endocrine pancreatic cancers could be classified with an accuracy above 80%. In primary colorectal cancers and patient matched liver metastasis, organized intratumor heterogeneity was discovered by MSI but both entities showed high degree of similarity and no evidence of metastasis forming clusters could be found. By sharing the complete analysis histories in Galaxy and additionally the raw data via the PRIDE repository when publishing, we make our MSI studies fully reproducible and transparent and advocate open science.

IL44

Monitoring chemoresponse and personalise treatment using patient derived ovarian cancer samples

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Chemoresistance remains the major barrier to effective treatment of ovarian cancer and predicting chemoresponse is an unmet clinical need. We developed a carboplatin resistant ovarian cancer cell line model and observed enhanced migratory and invasive capabilities in resistant cells. Proteomics analysis was able to separate these populations based on their molecular features. To be able to monitor direct response of patient derived samples to chemotherapy, we used patient derived ovarian cancer spheroids (3-dimensional multicellular cell clusters), which can be easily isolated from malignant ascites. We performed MALDI-mass spectrometry imaging on spheroids and were not only able to visualise three distinct layers (proliferating, quiescent, and necrotic) within the spheroids, but were also able to monitor drug response. For potential translation into a clinical setting, we developed an assay ready tissue culture plate, which needs minimal sample handling of malignant ascites and can be preloaded with chemotherapy combinations of choice. The chemotherapy response of the patient derived cells/spheroids can be easily monitored using standard fluorochromic stains. This is a very exciting example in the use of Proteomics and Mass Spectrometry Imaging to support personalised medicine and treatment of cancer patients in the future.

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Mittal P, Price ZK, Lokman NA, Ricciardelli C, Oehler MK, Klingler-Hoffmann M, Hoffmann P. Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI MSI) for Monitoring of Drug Response

in Primary Cancer Spheroids. Proteomics. 2019 Nov;19(21-22):e1900146. PMID: 31474002.

IL45

Towards a human data-driven reference kinase-substrate probabilistic network

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Phosphoproteomics data provide a snapshot of the phosphorylation-based signaling state of cells. They can therefore be used to dissect the dynamic networks active in a cell in a given condition. Several methods infer context-specific signalling networks from phosphoproteomics data by using as informative priors either existing protein-protein interaction networks or networks from pathway databases. These suffer from severe study bias and therefore data-driven analyses could provide more scope for novel discoveries and an improved understanding of context-specific cell signalling.

Here I will present a data-driven machine-learning-based approach that takes advantage of global phosphoproteomics datasets to predict kinase-kinase regulatory networks including their direction and sign of regulation. We further extended this method to include non-kinase substrates and provide predictions for at the phosphosite level. We were able to provide predictions for a large fraction of the understudied kinase space and found that kinase regulatory networks are denser than previously suspected.

Our methodology displays improved performance compared to other state-of-the-art kinase-substrate prediction methods and provides predictions for more kinases than most of them. Importantly, it better captures new experimentally identified kinase-substrate relationships. It can therefore allow the improved prioritisation of kinase-substrate pairs for illuminating the dark human cell signalling space.

IL46

Proteomics to study Candida albicans-host interaction: focus on stress and macrophage responses

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Candida albicans is a commensal fungus that causes systemic infections in immunosuppressed patients. In order to deal with the changing environment during commensalism or infection, C. albicans must reprogram its proteome. Characterizing the stress- and macrophages-induced changes in the proteome that C. albicans uses to survive should be very useful in the development of new antifungal drugs.

First, we studied the C. albicans global proteome after exposure to hydrogen peroxide (H2O2) and acetic acid (AA) and after interaction with human macrophages using a data-independent acquisition mass spectrometry (DIA-MS) strategy. C. albicans responded to treatment with H2O2 with an increase in the abundance of many proteins involved in the oxidative stress response, protein folding, and proteasome-dependent catabolism, which led to increased proteasome activity. Treatment with AA resulted in a general decrease in the abundance of proteins involved in amino acid biosynthesis, protein folding, and rRNA processing. Almost all proteasome proteins declined, as did proteasome activity. Apoptosis was observed after treatment with H2O2 but not with AA. A targeted proteomic study of 32 proteins related to apoptosis in yeast supported the results obtained by DIA-MS and allowed the creation of an efficient method to quantify relevant proteins after treatment with stressors (H2O2, AA, and amphotericin B).

Monitoring C. albicans proteome after interaction with THP-1 human macrophages at three time points showed both conserved and specific changes in proteins at each time point. Over time a decrease in cell wall, nucleus and mitochondrial proteins was observed. At 3 h, the proteomic changes showed a response to oxidative damage and a slight mitochondrial involvement. The most relevant aspect revealed after 6 h of interaction was an extensive decrease of mitochondrial proteins that was correlated

with a lower activity of this organelle. After 9 h of interaction, less changes in the protein abundance were observed when compared with previous time points, suggesting a recovery of C. albicans cells after escaping from macrophage. Of note, the discovery of Prn1 as a key protein in the defense against oxidative stress as well the increase in the abundance of Oye32 protein when apoptotic process occurred point them out as possible drug targets.

AL02

Effect of a diet rich in omega-3 fatty acid on acute-phase proteins and high-density lipoproteins in the plasma proteome of gestating and lactating sows: a sixteen-week intervention trial

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Objective: Diets rich in omega-3 fatty acids (FA) have been associated with anti-inflammatory effects, promoting health and performance. This study aimed at investigating the effect of increased dietary intake of omega-3 FA on the plasma proteome of sows from the end of gestation to the end of lactation. **Methods**: Sixteen multiparous sows were fed either soybean oil (containing a lower dose of omega-3, CR) or linseed oil (containing a higher dose of omega-3, LR) during gestation and lactation. Plasma samples (n = 5/group/time point) at day 108 of gestation (G108) and end of lactation (L-End) were digested with trypsin following the filter-aided sample preparation (FASP) protocol. Tryptic peptides were analyzed using a tandem mass tag (TMT)-based quantitative proteomics approach to reveal differentially abundant proteins (DAP) between treatment groups. An internal standard was prepared by pooling an equal amount of each sample to normalize signals between runs. Protein identification and quantification was performed using Proteome Discoverer platform (version 2.4, Thermo Fisher Scientific). Selected proteins showing consistently altered levels between groups were validated by Western blot for measuring apolipoprotein A1 (APOA1) and ELISA technique for measuring haptoglobin (HP) and Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4 or pig-MAP). **Results**: In total, 379 proteins and 202 master proteins were identified. In the LR group, the down-regulation of HP and Serum amyloid P-component (APCS) and up-regulation of alpha-1-antitrypsin (SERPINA1) may relate to the mechanism of anti-inflammation and protease inhibition. At L-End, the increased abundance of apolipoproteins such as APOA1, APOA2, and APOC3 and decreased abundance of positive acute-phase proteins such as HP and ITIH4 (or pig-MAP) is consistent with their involvement in the regulation of plasma lipoproteins and defence responses to stress stimuli.
Conclusion: These results provide additional insight into the benefit of increased dietary omega-3 FA in altering proteins that participate in defence systems against gestational and lactational stresses. Furthermore, the underlying mechanism that drives the sow"s stress response from late gestation to late lactation was revealed.

AL03

Metaproteomics investigation of goat cheese: ripening and safety concerns

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Objective:

Caprino Nicastrese is a typical Calabrian cheese, south Italy, with unique gustatory and olfactory essences resulting from the fermentative activity of the indigenous microflora of raw milk from Nicastrese bred goats [1,2]. To date, extensive studies on the microbiota harboured in this cheese are lacking and the dynamics of the microbial community over time and space are largely unknown. It is the aim of our study to elucidate the microbial community composition and activities in the diverse cheese wheel regions, at diverse ripening periods

Methods:

We integrated a microbial community fingerprinting technique (*i.e.* 16S rRNA gene sequencing) with a label-free shotgun metaproteomics approach to study the microbial community of the rind and core of the cheese wheel at 30, 60 and 90 days of ripening. Knowledge from the two independent approaches is integrated for a comprehensive survey of the microbial communities at the diverse ripening times and/or regions of the cheese wheel.

Results:

DNA-based featuring of the microbial community highlights a stable microbiota composition among the samples at the different ripening stages; whilst different composition of the microbial community is observed when comparing the core and rind samples of the cheese wheel. Accordingly, microbiota

composition assessed by the identified protein repertoire confirms the findings of the DNA-based microbiota composition assessment, although a higher bacterial diversity has been depicted by the metaproteomics approach. Specifically, highly abundant bacterial families such as *Lactobacillaceae* and *Streptococcaceae* are commonly identified, but discordant results are registered regarding the less abundant bacterial families (*i.e. Paenibacillaceae* and *Moraxellaceae*). Functional featuring of the microbial communities highlights the different functional concerns of the microbial communities harbouring the rind or the core of the cheese wheel. The rind microbiota is predominantly focused on aerobic metabolism whilst the cheese core microbiota is involved in other biochemical pathways such as DNA methylation and protein refolding.

Proteins involved in bacterial competition and biosafety maintenance are also highlighted in the core microbiota.

Conclusion:

Altogether, these observations provide pivotal knowledge for delivering guidelines and/or amending cheesemaking procedures aimed at cheese standardization and ensuring the microbiological safety of the artisanal products.

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This work was supported by PON-FSE, grant number AIM1879147 – 2.

Authors are grateful to Dr. Floro De Nardo for the precious guidance through the Nicastrese breed.

AL04

Biomarkers for diagnosing the retained placenta in cows-does the analysis of serum proteome keep the secret?

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Question: Retained placenta (RP) is a common disorder during the puerperium in dairy cows with a high negative impact on the health condition of the cows and the milk production. The initiation of the RP therapy when the clinical signs are already present has low effectiveness, which highlights the necessity for the biomarkers that would indicate the risk for the RP before the occurrence of the signs. The study aimed to investigate the serum proteome as the source of the biomarkers for diagnosing the RP.

Methods: The study included nine cows with the RP and six with the physiologic puerperium. The serum proteins were reduced, alkylated, precipitated with acetone, digested with trypsin, and labeled with the tandem mass tags. The liquid chromatography-tandem mass spectrometry platform consisted of the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptides were separated using the linear gradient and scanned in the positive ion mode using the data-dependent analysis Top8 method. For the identification and relative quantitation following the SEQUEST algorithm, the Proteome Discoverer® software was employed. Further, the data were analyzed with the R statistical package and Protein Analysis Through Evolutionary Relationship (PANTHER) classification tool.

Results: In total, 651 proteins were quantified. Fifty-four had different relative abundance in the cows with RP and those with the physiological puerperium, whereby approximately two-thirds were more abundant in the RP cases. The main classes containing the proteins with the different abundances were the modulators of protein-binding activity and the protein modifying enzymes. The gene ontology results indicated that these proteins posse catalytic activity, binding, and regulatory functions in eleven biological

processes. The highest increase in abundance was for Haptoglobin, followed by lipopolysaccharide-binding protein, inter-alpha-trypsin inhibitor heavy chain, and glutathione peroxidase. The cows with RP had the highest decrease in apolipoprotein A-IV, while it was less protruding for C-type lectin domain family 3 member B, tetranectin, and serpin family D member 1.

Conclusion: The serum proteome analysis deciphered several aspects of the RP molecular pathology. Also, it identified several proteins with the potential to serve as the RP biomarkers after further clinical validation.

AL05

Application of fecal metaproteomics for routine monitoring of human gut diseases

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Objective: Studying the human gut microbiome while several diseases such as inflammatory bowel disease (IBD) or colon cancer revealed correlations between the diseases and alterations of the taxonomic and functional composition of the gut microbiome. Although it is not clear for all diseases, whether the microbiome dysbiosis is the cause or the results of a certain disease, monitoring the gut microbiome has a significant diagnostic potential. Fecal samples representing the conditions inside the intestine can be taken non-invasive even from patients. Thus, analysis of the fecal microbiome could be used for disease diagnosis, control of therapy or disease progression, and for preventive medical check-ups. In contrast to amplicon sequencing and metagenomics that are frequently used to analyse the gut microbiome, metaproteomics provides structural and, in particular, functional data by identification of microbial and host proteins. The latter provide information about the immune system and digestive organs of patients including the integrity of the gut barrier.

Methods: For the analysis of fecal samples, we developed a standardized and fast metaproteomics workflow that comprises cell lysis, protein extraction, tryptic digestion and LC-MS/MS measurements. Use of the MetaProteomeAnalyzer allows bioinformatic data evaluation. The workflow enables the analysis of single samples within one day and could also be parallelized in 96 well-plates.

Results: We applied this workflow on samples from patients with IBD, non-alcoholic fatty liver (NASH), and after a weight-loss targeting a better diagnosis and therapy monitoring. Fecal samples contained larger amounts of human proteins for patients than for healthy individuals indicating pathogenic alterations. In particular, the inflammation marker calprotectin (protein S100A8/A9) was increased in IBD and obese patients. Furthermore, human alpha-amylase was elevated in obese patients and spondin in NASH patients. Microbial proteins and taxon abundance seemed to correlate mainly with the diet. In the case of IBD patients, successful therapy resulted in a decreased abundance of human proteins and increased abundance of protein involved in microbial hydrolysis.

Conclusion: In sum, fecal metaproteomics based on microbial and human proteins can be applied to differentiate between healthy and diseased patients. Thus, it may be useful to apply metaproteomics in routine analysis of the patients" microbiomes to diagnose and monitor disease progression.

AL06

Characterization of dynamic profiles of the respiratory microbiota and host response from cystic fibrosis sputum samples

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Objective. Cystic fibrosis (CF) is a polymorphic disease marked by multiple and difficult-to-treat respiratory exacerbations. The lung microenvironment, including its microbiota, are potential driving causes for the worsening of patient symptoms and the variability in the treatment outcome. This study was designed to examine the dynamic profiles of the respiratory

microbiota, its microbial composition and activities, and the concomitant host response. Multiple metaproteomic measurements were taken using sputum samples collected longitudinally from several CF patients chronically colonized by nontuberculous mycobacteria (NTM) with various antibiotic treatment regimes and disease outcomes.

Methods. For each CF sample, the extracted peptide mixture was injected for analysis by shotgun tandem MS on a Q-Exactive HF mass spectrometer operated in a data-dependent mode. The taxonomical and functional composition of the microbiota and the host-related functions were obtained via an optimized multi-round search process using the Mascot Daemon search engine and the in-house developed morg.id tool. PSM validated with a Mascot p-value of 0.05 during the last-round search were filtered using an FDR < 1% and subsequently used to infer peptide and protein identifications. Proteins were grouped if they shared at least one peptide. Label-free quantification was performed based on PSM counts for each protein, applying the principle of parsimony. For each phylum, spectral counts for all the proteins in a group were summed to assign abundance values to each protein group. Proteins were KEGG-annotated using the GhostKoala web service. The spectrum count values for peptides mapped to a KEGG through protein mapping were summed to attribute an abundance value to each functional term. Peptide-to-taxon mapping was also performed to allow taxon-resolved functional quantification.

Results. An overall average of 57,814 ± 4,333 MS/MS was obtained per sample. A multiple-step cascaded search allowed 31 ± 4% of spectra to be assigned per sample. The distribution of the assigned Taxon-Spectrum Matches as a function of their origin revealed that metaproteomics allows monitoring host responses in addition to the dynamics of the microbial profiles. Preliminary results show differences in the microbiota profiles for samples following different antibiotic treatments, as further confirmed by a peptide-based functional metaproteomics analysis. These analyses revealed mechanisms that potentially confer a competitive advantage to NTM. Similarly, host molecular functions linked to inflammation and immune response were associated with an increased abundance of opportunistic pathogens.

Conclusion. Overall, metaproteomics provides a unique approach to studying the CF respiratory microbiome. Applied to a larger number of samples, this approach holds the potential to identify innovative measures for controlling infection and/ or guide future clinical diagnosis.

AL07

Functional alterations induced by bisphenols in a simplified human intestinal microbiota (SIHUMIx)

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Bisphenols are industrial chemicals used in the process of polymerization of polycarbonate plastics and epoxy resins. After partially banning Bisphenol A (BPA) in certain consumer products, Bisphenol S (BPS) and Bisphenol F (BPF) are widely used as alternatives. Based on the variety of products used in our everyday life, we are exposed to various chemicals through the skin or diet. The crucial role of the gut microbiome in digesting, energy and vitamin provision can be thrown out of balance by diet, lifestyle changes or non-naturally derived chemicals. Associated with several diseases, the disbalance of the microbiome is a gateway for altered functionality. Leading towards realistic exposure, combined environmental chemicals should be of high concern in risk assessment.

In this study, we are investigating the effect of chemicals and their mixtures on the gut microbiome in models of differing complexity. Starting from single strain exposure in batch culture towards an established simplified human intestinal microbiota (SIHUMIx) in an *in vitro* continuous cultivation system. *Bacteroides thetaiotaomicron*, a highly abundant and mucin degrading commensal human bacterium, was selected as representative for single chemical testing. Grown in batch it was exposed from beginning to late exponential phase to a range of concentrations of BPA (0-0.6 mM), BPS (0-6mM) and BPF (0-2 mM) individually. Membrane fatty acid composition was monitored by fatty acid methyl ester (FAME) analysis to investigate membrane alterations. Additionally, BPF was selected in concentrations of 113 µM and 565 µM

to investigate changes in the proteome and short chain fatty acid (SCFA) production. In a second experiment, SIHUMIx was grown for 7 days in bioreactors building a stable community, then chemicals were added over 8 days, followed by an 8 day recovery phase. Bioreactor vessels were supplied with environmental relevant concentrations of BPS and BPF (14 µM each) during exposure phase. Changes in functional profiles were analyzed by metaproteomics and metabolomics.

We observed increasing inhibition of growth in more hydrophobic bisphenols in concentration dependent manner. *Bacteroides thetaiotaomicron* showed an increase in saturated fatty acids and acetate production during BPF treatment, assuming a stress reaction towards chemicals. Membrane adaption was reflected at the proteome level by an increase in several transporters associated in cell wall biogenesis. Sensitivity to chemical exposure was greater in gram-positive than gram-negative bacteria as observed in SIHUMIx. Alterations induced by chemical treatments were reversible as monitored in the recovery phase.

Examining functional changes of bacterial gut communities as a result of environmental chemicals will allow a more realistic approach in risk assessment. Focused on increasing complexity by combining multiple chemicals and scaling up microbial communities will be a considerable step in microbiome-disease research.

AL08

Multi-layered chromatin proteomics identifies cell vulnerabilities in DNA repair

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The DNA damage response (DDR) is essential to maintain genome stability, and its deregulation predisposes to carcinogenesis while encompassing attractive targets for cancer therapy. Elective and most successful example is the selective sensitivity of homologous recombination (HR)-defective cancers for inhibitors of epigenetic regulators impinging on the post-translational state of chromatin, such as the Poly [ADP-ribose] polymerase (PARP). Chromatin governs the DDR *via* the tight interplay among all different chromatin layers including DNA accessibility, histone post-translational modifications (hPTMs), and chromatin-associated proteins. Yet, the full composition of these layers and the functional interaction between them is only partially known, and they have not been systematically investigated to identify novel vulnerabilities in cancer cells undergoing double-strand break repair.

Therefore, we here establish and employ a multi-layered proteomics-based approach to quantify and dynamically profile different chromatin layers at high temporal resolution during DNA double-strand break repair. In particular, we **a**) use ChIP to enrich for known repair proteins and characterize their functional chromatin-mediated interactions *via* the Selective Isolation of Chromatin-Associated Proteins (or ChIP-SICAP), **b**) dissect the composition of the DNA-bound proteome by the isolation of Proteins on Chromatin (or iPOC), and **c**) profile signatures of hPTMs at mononucleosome resolution with Native Chromatin Proteomics (N-ChroP).

Through our multilayered approach we quantify a high number of both expected and novel determinants deregulated during the DDR, and we validate our proteomic data by means of imaging- and FACS-based orthogonal approaches. All together, these results allow us to functionally attribute novel chromatin-associated proteins to repair by either non-homologous end-joining or HR, thus revealing a role for the histone reader ATAD2, the microtubule organizer TPX2 and the histone methyltransferase G9A as important regulators of the HR repair. Our data also show how depletion of these chromatin regulators sensitizes to PARP inhibitors, thus paving the way to innovative combinatorial epigenetic drug treatments. Furthermore, during the DDR we dynamically profile 33 hPTMs at mononucleosomes bearing the DNA damage marker gamma H2AX (or γH2AX). Integration of these data with the complementary results from iPOC implicated G9A-mediated monomethylation of H3K56 in homologous recombination repair. Collectively, we provide an unprecedented and dynamic chromatin-centered proteomic view of the DDR, while representing a valuable resource for the use of PARP inhibitors in cancer.

https://www.biorxiv.org/content/10.1101/2021.08.05.455226v1

Fig. 1

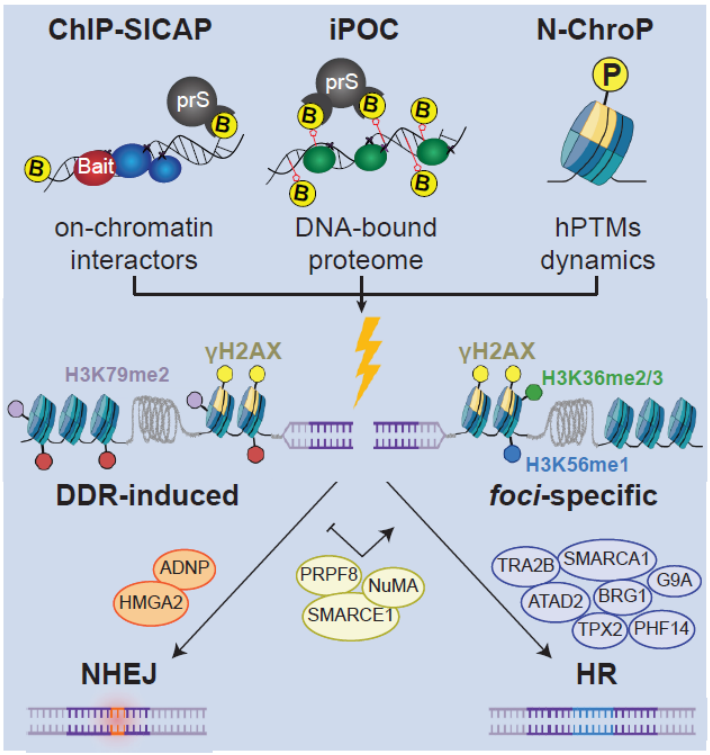
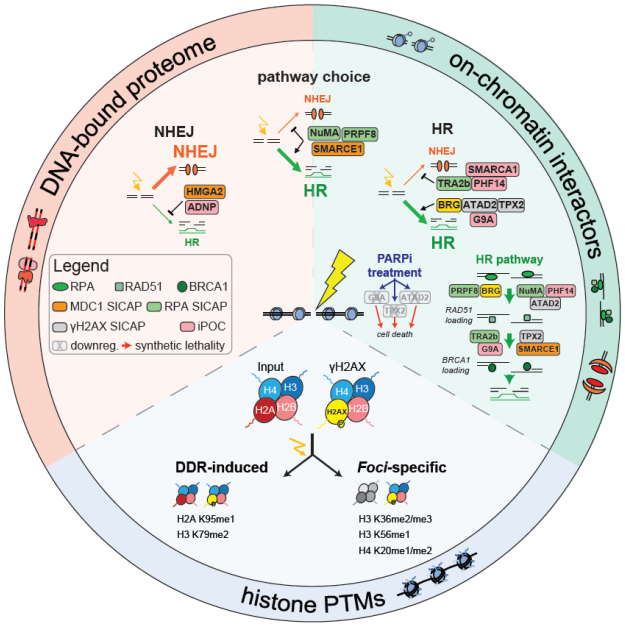


Fig. 2



AL09

Investigating mutated phosphorylation sites within intrinsically disordered regions using a peptide-based interaction screen

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With the recent advances in sequencing technologies, the number of identified pathogenic mutations has increased drastically and surpassed our ability to functionally characterize them. Many disease-causing missense mutations affect intrinsically disordered regions (IDRs). Since these regions do not adapt a defined 3D structure, it is not clear how these mutations affect protein function. IDRs often contain short linear motifs (SLiMs) that mediate interactions with other proteins and/or are substrates of posttranslational modications such as phosphorylation. As SLiMs are crucial for protein-protein interactions (PPIs), studying how mutations in IDRs alter PPIs can provide information on disease mechanisms. Here, we employed a peptide-based screen to assess how pathogenic mutations of known phosphorylation sites affect PPIs. To this end, we selected 38 disease causing mutations, germline and somatic, from the PTMVar database of PhosphositePlus. Peptides corresponding to the mutated regions were synthesized on cellulose membranes in the wild-type, mutated and phosphorylated form and used to pull-down interacting proteins from cellular extracts. We find that the interactome of the phosphorylated and mutated peptides differed markedly, with most of the interactions being lost upon mutation, in several cases due to destruction of a phosphorylated SLiM. We have further used proximity labeling-mass spectrometry, BioID, to validate some of the screen results. Both the screen and BioID results imply a role of SYNRG in clathrin-mediated endocytosis, a result in concordance with the literature. Moreover, we observed that a phosphorylated peptide derived from the zinc-finger protein GATAD1 specifically interacts with 14-3-3 proteins while the corresponding mutated peptide does not. The mutation of this phosphorylation site causes dilated cardiomyopathy. Knowing that 14-3-3 proteins bind to phosphorylated proteins to retain them in the nucleus, a disruption of GATAD1/14-3-3 interaction could lead to GATAD1 mislocalisation. In

summary, our data indicates that peptide-based interaction screens provide unique insights into the function of phosphorylation sites in health and disease.

AL10

Combining infrared spectroscopy and mass spectrometry for comprehensive analysis of amyloid-β peptides

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Objectives: Amyloid-β (Aß) peptides from liquid biopsies, such as cerebrospinal fluid (CSF) or plasma, exhibit abnormal characteristics related to Alzheimer's disease (AD) development, including endogenous concentrations and structural conformation. Therefore, they play a central role as biomarkers in the diagnosis of the disease. Immuno-infrared spectroscopy has shown that the distribution of Aß peptide secondary structure serves as a highly specific biomarker feature at an early stage of AD (Nabers A. et al., 2016). In this work, we present the combination of mass spectrometry (MS) with an immuno-infrared sensor technology that provides a multi-level assay for a comprehensive Aß peptide profiling.

Methods: A step-by-step workflow was developed for coupling mass spectrometry to the immuno-infrared sensor. It includes a specific and MS-compatible protocol for the elution of the sensor-bound Aß peptide fraction, subsequent sample preparation for MS analysis, and global as well as targeted MS methods for Aß peptides. The latter includes the development of a rapid top-down direct infusion (DI)-MS approach for the quantification of Aß peptides.

Results: A workflow for combining MS with the immuno-infrared sensor technology was established and was successfully applied for CSF analysis. The Aß DI-MS approach was developed that improves Aß peptides quantification and allows a reduction in MS analysis time down to 2 minutes. The linear detection range of the DI-MS method for Aß 1-40 and Aß 1-42 was 1-50 fM/µl with R2= 0.96. The obtained results were compared with a standard immunoprecipitation (IP)-MS approach and showed good agreement. In Brief, Aß 1-40, Aß 1-42 as well as known Aß-binding proteins, were identified in the eluates from both the sensor and the IP-MS. The senor-based MS results were furthermore correlated with the infrared spectroscopy results.

Conclusions: The combination of immuno-infrared sensor technology and mass spectrometry analysis offers high potential to provide added value for improving AD diagnosis and furthermore to gain new insights into the molecular network of Aß at the protein/peptide level.

AL11

Dynamic SILAC-PTM-MS reveals wiring of phosphorylation during protein maturation

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Objective

Post-translational modifications (PTMs), such as reversible phosphorylation, control all aspects of protein function. Despite advances in the identification and localisation of PTMs by mass spectrometry (MS)-based omics technologies, much of current understanding of PTMs is lacking the temporal dimension. Specifically, how (or whether) PTMs are added and removed at specific times over a protein"s lifetime from its synthesis, folding, maturation and function, through to its eventual degradation, has so far remained largely elusive.

Methods

We combine two-label dynamic stable isotope labelling of amino acids in cell culture (SILAC) with phosphoenrichment of peptides to create dynamic SILAC-PTM-MS and use it to quantify the apparent turnover on the modified peptide level. Using quantitative modelling we generate a theoretical framework for dynamic SILAC-PTM-MS and analyse the

relationship of apparent peptide turnover to kinetics of PTM addition/removal and protein degradation. Finally, we test our predictions from modelling using rational mutagenesis of phosphosites identified by dynamic SILAC-PTM-MS.

Results

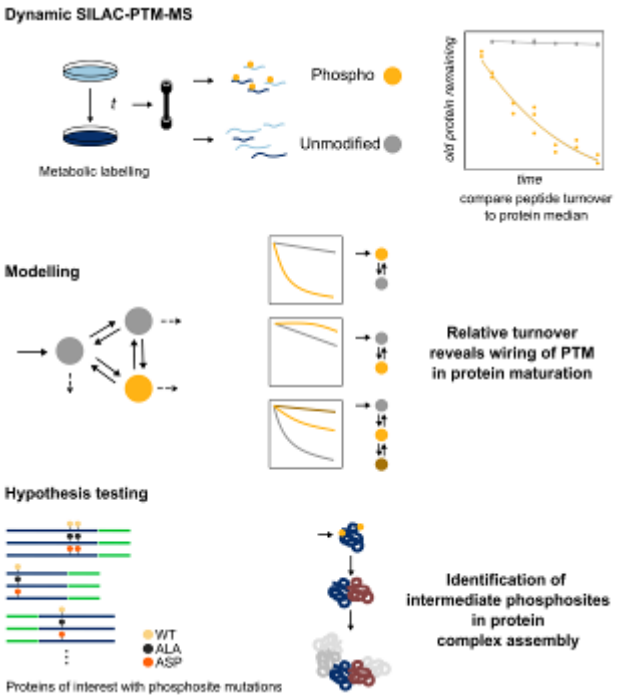
From mathematical modelling we predict that the relative apparent turnover of a protein subspecies (such as a proteoform carrying a PTM) compared to the total pool of that protein is primarily defined by the kinetics of the formation of said proteoform (e.g. the addition/removal of a PTM), as well as the order of its formation with respect to protein synthesis—and thus not a direct measure of proteolytic stability.

This prediction is confirmed by our experimental results showing expected fast apparent turnover of intermediate peptides in known protein maturation events, such as protein N-terminal acetylation. We also find that peptides with fast apparent turnover are strongly enriched for carrying other PTM sites suggesting that they represent intermediates in ordered PTM cascades.

We test our prediction that fast apparent turnover does not predict low proteolytic stability by mutagenesis of 66 phosphorylation sites on 24 proteins identified in our proteomic experiment. In accordance with our modelling, we find that the vast majority of mutations thus tested do not change a protein"s proteolytic stability. Instead, we verify a previously-unannotated phosphorylation site on a proteasomal subunit predicted by dynamic SILAC-PTM-MS to represent a maturation intermediate, and find that it is likely involved in the assembly of the proteasome.

Conclusion We present dynamic SILAC-PTM-MS: a combination of dynamic isotopic labelling and PTM-specific enrichment. Leveraging insight from theory, we show that dynamic SILAC-PTM-MS can identify PTM sites involved in protein maturation. We present a rich, proteome-wide dataset of phosphorylation sites of interest including, e.g., a site involved in assembly of the proteasome.

Fig. 1



AL12

Regulation of lysosomal proteins by phosphorylation

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Background

Lysosomes are membrane-bound organelles that contain >60 different hydrolases. Lysosomes are considered as a center of cellular metabolism, monitoring nutrient availability and coordinating anabolic/catabolic signaling. The major location for this regulation is the lysosomal membrane, the most important regulator being the mammalian target of rapamycin complex 1 (mTORC1). While signals transmitted from the lysosome to the cell are well known, it is not well understood whether mechanisms signal towards lysosomal proteins. Therefore, we sought to examine if the phosphorylation status of lysosomal proteins can be dynamically modified in response to a stimulus influencing lysosomal function.

Methods

To impair lysosomal function, we employed U18666A, a widely used inhibitor of the lysosomal cholesterol transporter NPC1. The treatment was validated by Western blotting and immunostaining. We performed SILAC-based proteomic and phosphoproteomic, as well as metabolomic and lipidomic analyses of samples extracted from both control and treated mouse embryonic fibroblasts. For follow-up studies, Lamtor1 co-immunoprecipitation (Co-IP) and enrichment of lysosomes were performed, followed by LC-MS/MS analyses.

Results

In total, ~7,000 proteins and ~13,000 phosphorylation sites were identified in three independent biological replicates containing lysosomal and lysosome-associated proteins. In the proteomic dataset, out of the 87 lysosomal proteins identified, 4 were found to be regulated. In the phosphoproteomic dataset, 28 lysosomal or lysosome-associated proteins were phosphorylated, of which 10 were regulated. The lipidome analysis identified >150 lipids of which cholesterol esters were found to be regulated, demonstrating global effects of cholesterol transport inhibition of the lipidome of cells. Furthermore, the metabolomic analysis revealed a significant decrease in the levels of carnitine. Based on the phosphoproteomic dataset, we selected a subset of differentially phosphorylated lysosomal and lysosome-associated proteins for a detailed examination by the generation of mutant versions. Among the selected candidates, phosphorylation of Lamtor1 affected the protein stability and interaction with members of mTORC1 machinery. To investigate the kinase responsible for Lamtor1 phosphorylation, co-immunoprecipitation and lysosome enrichment were performed, followed by LC-MS/MS analysis. In the co-immunoprecipitated and lysosome-enriched samples, 64 and 7 kinases were overrepresented compared to control samples, respectively. From the obtained dataset, several kinases were selected for further studies.

Conclusion

We present the first proteomic, phosphoproteomic, metabolomic, and lipidomic analysis of altered cholesterol metabolism, identifying regulatory effects on different levels and providing detailed follow-up analysis on Lamtor1.

AL13

Detecting and quantifying rare translational errors by data-independent acquisition

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Objective

During mRNA translation, ribosomes can change the reading frame by slipping on the mRNA. This ribosomal frameshifting results in the synthesis of a completely different amino acid sequence – a non-canonical protein form. It is thought be rare (once in 1 000 – 10 000 codons) and occurs stochastically, and so these proteins often go unnoticed by conventional analyses. So far, there is no proteome-wide data on where and how often frameshifting happens. Thus, they form a part of the "dark proteome" in mass spectrometry – peptides not matching canonical protein forms. By combining a customized sequence database of predicted frameshift sequences with high-coverage data-independent acquisition (DIA), we aim to identify and quantify peptides resulting from frameshift events and determine the exact type, position and frequency of frameshift errors.

Methods

We applied a DIA scheme geared towards high specificity with narrow, overlapping isolation windows. Via multiple injections per sample, each covering a small m/z range, we simultaneously maximized coverage. Based on the canonical coding sequences we predicted the protein sequences resulting from a ribosomal frameshift at any codon position. This customized database was set up so that each sequence could be associated with a particular error type and position. The spectra were searched with the DIA-NN software against the custom-built frameshift database together with the reference Uniprot proteome and sequences of common protein contaminants. Identified precursors were filtered for detection in multiple replicates and a strict q-value cutoff, estimating false positive rate by the two-species hybrid method.

Results

Our method identified more than 4.500 canonical *S.cerevisiae* proteins (ca. 75% of the proteome) with over 40 000 matched peptides, as well as over 100 potential frameshifted peptides that were further confirmed by manual inspection of spectra. They did not match to any canonical sequences in the whole Uniprot database or the six-frame translation of the yeast genome and cannot be attributed to single amino acid substitutions. We thus conclude to have identified genuine frameshifting events. To determine the frequency of specific frameshifting events, we are now quantifying frameshifted peptides and their corresponding source proteins using targeted AQUA, FUGIS and MS Western approaches.

Conclusion

We present a generic strategy for comprehensive characterization of frameshifted peptides in eukaryotes, which constitute a poorly predictable and elusive fraction of the dark proteome. This general approach can be extended to detect other non-canonical peptides to provide a resource of novel protein forms generated by translational errors, as well as to determine their frequencies and absolute abundances.

AL14

Connecting (phospho)proteomics and drug phenotypes uncovers the modes of action of kinase inhibitors in sarcoma cells

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Objective

Understanding the cellular mode of action (MoA) of drugs is essential for precision medicine. Combining omics technologies and drug response measurements has proven useful in this regard. Kinase inhibitors (KIs) are important cancer drugs but often display polypharmacology that is not molecularly understood. To approach this systematically, we combine full- and phospho-proteomic analysis and high-throughput phenotypic drug screening to study the interactions of 150 clinical kinase inhibitors and signaling pathways in sarcoma cells.

Methods

The proteomes and phosphoproteomes from 17 sarcoma cell lines (12 entities) were characterized by micro- and nano-LC-MS/MS. High-throughput cell viability assays were performed on these cell lines in 384-well plates for 150 cancer drugs, 139 of which are kinase inhibitors. The activity landscape of 17 cell lines was calculated by integrating the kinase abundance, kinase phosphorylation, and substrate phosphorylation. The abundances of protein and phospho-sites were further integrated with the drug responses by simple correlation and elastic net regression.

Results

We present deep (phospho)proteomic profiling consisting 9,200 to 10,500 proteins and 10,300 to 27,100 phospho-sites per cell line as well as 2,550 phenotypic drug profiles, covering broad signaling landscapes and distinct drug responses in sarcoma respectively. We showed that the kinase-based activity landscape often explained the drug sensitivity in our screening. The connectivity between (phospho)proteomics data and drug responses further revealed known and novel MoAs of many drugs. For data mining by the scientific community, we provide an interactive web application.

Conclusions

Our study provides a rich resource for (1) understanding active signaling pathways in sarcoma cells, (2) identifying treatment response predictors, and (3) revealing novel MoA of clinical KIs and non-KIs. These findings serve as the first step to building a knowledge base for precision medicine.

AL15

Pharmacoproteomics of cerebrospinal fluid establishes biomarkers for precision medicine of Alzheimer's disease

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Proteome analytics of cerebrospinal fluid (CSF) may identify biomarkers to diagnose a brain disease or biomarkers to measure treatment responses in a precision medicine approach. One major brain disorder is Alzheimer"s disease (AD), for which the protease BACE1 is a key drug target. However, BACE1-targeted inhibitors in phase 3 clinical trials showed psychiatric and cognitive side effects, which are assumed to be mechanism-based and to be caused by too strong inhibition of cleavage of multiple BACE1 substrates in the brain. CSF proteomics may determine which BACE1 substrates are detectable in CSF, respond to BACE1 inhibition and may cause the side effects.

We used label-free shotgun CSF proteomics to identify in vivo BACE1 substrates from minute amounts of CSF from mice, non-human primates and humans dosed with a BACE1 inhibitor. Across species, BACE1 inhibition reduced cleavage of several new and known BACE1 substrates in a time- and dose-dependent manner, including SEZ6, SEZ6L, CACHD1, CD200 and VCAM1. This analysis established a proteomic CSF fingerprint for in vivo BACE1 activity. Correlation of the pharmacoproteomic fingerprint to the occurrence of side effects in the clinical trials suggests that dose-lowering to less than 50% BACE1 inhibition may be an easy way to prevent side effects while maintaining efficacy for treating or prevening AD.

Taken together, our study demonstrates that CSF pharmacoproteomics is a powerful approach to mechanistically understand drug-induced side effects and may provide recommendations for precision medicine, such as targeted dose adjustment for each patient.

AL16

Proteomic basis for understanding the combination of gemcitabine and kinase inhibitors to kill pancreatic cancer cells

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Objective: 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 (chemo-)proteomics to characterize the combination of gemcitabine and kinase inhibitors in PDAC cells on a molecular level.

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.

Conclusion: 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 in the future.

(1) Bian, Y. et al. 2020 *Nat Commun* 11, 157

AL17

Thermal proteome profiling with compound concentration range exhibits the potential to probe protein-protein interactions in living cells by small molecule arranged thermal proximity coaggregation (smarTPCA)

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Objective:

Chemical biology and the application of small molecules has proven to be a potent perturbation strategy especially for the functional elucidation of proteins, their networks and regulators. In recent years, the cellular thermal shift assay (CETSA) and its proteome-wide extension, thermal proteome profiling (TPP), have proven to be effective tools for identifying interactions of small molecules with their target and off-target proteins in living cells. Here, we suggest a small molecule arranged thermal proximity coaggregation (smarTPCA) assay, a method that was developed to exploit off-target effects for the characterization of protein-protein interactions in living cells.

Methods:

CETSA with single protein quantification by immunoblotting and TPP with proteome wide protein quantification by multiplexed (TMT 10plex) quantitative mass spectrometry (MS) were applied to detect thermal protein stabilization by the MAPK14 kinase inhibitors AMG-548 and SB203580. Temperature range (TR) as well as compound concentration range (CCR) studies were performed in living HL-60 cells as well as in HL-60 cell lysate. MS analysis was performed on Orbitrap Mass Spectrometers (Fusion Lumos Tribrid or Q Exactive) and data analysis was performed using MaxQuant and R scipts.

Results:

Using an established CETSA protocol, we not only found thermal stabilization of MAPK14 in living cells by AMG-548 and SB203580, as described previously, but we extended our analysis to MAPKAPK2 and MAPKAPK3, the best-described interaction partners and phosphorylation targets of MAPK14, which, we found, were also stabilized by the two inhibitors. Interestingly, although the melting curves and extent of inhibitor-induced thermal stabilization of MAPK14, MAPKAPK2, and MAPKAPK3 differed, their inhibitor-induced dose-response characteristics exhibited high similarity.

By extending our study to the proteome level using TPP-CCR and performing cluster analyses of filtered dose-response data, we confirmed the similar dose-response characteristics of MAPK14, MAPKAPK2, and MAPKAPK3. In addition, we found that other proteins, including myosin light chain kinase, smooth muscle (MYLK) with the highest confidence, also showed similar dose-response behavior to MAPK14.

Conclusion:

We conclude that the direct AMG-548 and SB203580 target MAPK14 shows thermal stabilization by direct interaction to the inhibitors and that MAPKAPK2 and MAPKAPK3 are co-stabilized in complex with MAPK14 resulting in highly similar dose response characteristics. Based on our finding, we support the idea that direct binders generally can be distinguished by their different inhibitor specificity profile, while co-stabilized proteins should closely resemble the inhibitor specificity profile of the direct binder.

We think that our smarTPCA method exhibits the potential to characterize protein-protein interactions in living cells by small molecule perturbation.

AL18

Building a Complete Glycoproteomics Analysis Workflow with MSFragger in FragPipe

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Introduction

Advances in methods for enrichment and mass spectrometric analysis of intact glycopeptides are increasingly producing large-scale, high-quality glycoproteomics datasets, but confidently annotating both peptide and glycan identities in the resulting spectra remains challenging. Advances in computational methods for glycopeptide identification, control of false discovery rates, and quantification are needed.

Methods

We have developed a pipeline for glycoproteomics analysis in the FragPipe environment using several software tools for fast, sensitive, and accurate annotation of glycopeptides from tandem mass spectrometry data. MS data is analyzed with MSFragger glyco search, which identifies glycopeptides as a peptide sequence and associated glycan mass, including the glycosylation site for singly modified N-glycopeptides. Next, we have developed a method for glycan composition determination with associated FDR control in the PTM-Shepherd post-search annotation tool, which converts the associated glycan mass to a specific composition. Finally, identified glycopeptides can be quantified in either TMT or label-free modes using quantitation tools TMT-Integrator or IonQuant, respectively.

Results

We show that the peptide-first search method of MSFragger glyco is fast and highly sensitive, capable of annotating many more glycopeptide spectra than competing tools at a given false discovery rate for both N- and O-linked glycopeptides. For N-glycopeptides, we show the composition assignment and glycan FDR filtering in PTM-Shepherd reports accurate glycan compositions, even in the presence of entrapment glycans known not to be present in the tested samples. In comparison to pGlyco3, another state-of-the-art glycopeptide analysis package, we demonstrate improved control of glycan composition FDR in yeast and mouse-derived glycopeptides, particularly in controlling unlikely matches to fucosylated glycans, while maintaining a dramatic improvement in the total number of glycopeptide spectra annotated at a given false discovery rate. Quantification of glycopeptides in TMT or label-free modes is similar to methods for typical proteomics analysis but incorporates the identified glycan information. We have adapted TMT-Integrator"s summary reports to include an additional report at the glycan level, allowing the abundances of individual glycans at a given glycosite to be compared across states when appropriate, or collapsed to glycosite, peptide, protein, or gene levels to support a wide range of analyses.

Conclusions

We present a complete workflow for identification of glycopeptides in FragPipe, including rapid and sensitive search, peptide and glycan FDR control, and quantitative tools for TMT and label-free modes, to support a wide range of glycoproteomics analyses.

AL19

Investigation of Post-translational Modification Isoforms of Transcription Factor EB (TFEB)

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Objective

Post-translational modifications (PTMs), such as phosphorylation, and acetylation, crucially affect the function, structure, and subcellular localization of proteins. Transcription factor EB (TFEB), a member of the microphthalmia (MiT) family of transcription factors, is known as the master regulator of autophagy and lysosomal biogenesis. Lysosomes are the main degradative organelles of cells are a central point for metabolic signaling. For TFEB, a connection between phosphorylation and localization has been established. In this context, the nutrient-dependent regulation of mammalian target of rapamycin complex 1 (mTORC1) is a major factor. mTORC1 phosphorylates TFEB, regulating its subcellular localization and hence transcriptional activity. While >20 phosphorylation sites of TFEB have been reported, only few have been thoroughly studied and no systematic investigation has been conducted for most of them.

Methods

TFEB subcellular localization was examined by microscopy, subcellular fractionation, and western blotting. TFEB was enriched from NIH/3T3 cells overexpressing TFEB-3xFLAG by immunoprecipitation and 2D gel electrophoresis was employed to investigate individual isoforms. For mass spectrometric (MS) analyses, TFEB was enriched from cytosolic and nuclear fractions, followed by SDS-PAGE. Gel bands of different molecular weight regions were in gel-digested using trypsin, chymotrypsin, and AspN. TFEB PTMs were identified by phosphopeptide enrichment, data-dependent acquisition (DDA) MS analyses and database searching using Mascot in combination with Proteome discoverer. Based on the identified peptides, a parallel reaction monitoring (PRM) assay was established on an Orbitrap Fusion Lumos; scheduling and data analysis were performed using Skyline.

Results

Microscopic investigation of NIH/3T3 cells, as well as western blot analysis of fractionated samples revealed a subcellular translocation of TFEB upon amino acid and lipid starvation, inhibition of mTORC1, and (in)activation of mitogen-activated protein kinase 1 (MAPK1). 2D gel electrophoresis revealed >30 isoforms of TFEB. MS analysis of unfractionated TFEB digests with/without phosphopeptide enrichment and data analyses by Mascot and error-tolerant searches revealed a total of 41 TFEB PTMs (34 phosphorylation and 7 acetylation sites). Based on these data, individual PRM assays were designed for trypsin, chymotrypsin and AspN, covering 50/99/70 target peptides, and 12/21/15 phosphorylation sites, respectively; together covering 94% of the TFEB protein sequence and 34 phosphorylation sites through 219 targets. TFEB was enriched from cytosolic and nuclear fractions of treated cells and analyzed with scheduled PRM assays of 120 min gradients, each.

Conclusion

In-depth analysis of TFEB identified 41 PTMs, some of which are novel, and >30 isoforms. Detailed PTM characterization by PRM allows for the investigation of molecular mechanisms regulating TFEB"s transcriptional activity.

AL20

The dual role of PRMT1-dependent arginine methylation in multi-layered gene expression regulation in response to genotoxic stress in cancer

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Arginine (R)-methylation is a protein post-translational modification implicated in the regulation of several cellular processes, including transcription, RNA splicing and DNA damage response (Guccione and Richard, 2019). R-methylation is catalyzed by the members of the protein arginine methyltransferase (PRMTs) family, of which PRMT1 is the predominant enzyme. The recent optimization of various MS-proteomics workflows has led to the expansion of the experimental R-methyl proteome annotation and to a better understanding of its plasticity in response to external stimuli.

Recently, we investigated the role of PRMT1-mediated R-methylation in response to the genotoxic stress induced by cisplatin (CDDP) in ovarian cancer (Musiani et al., 2020). We detected methylation changes in 92 R-sites on 56 proteins, with an overall methylation increase of histone H3 and chromatin-associated proteins, which was mirrored by the hypo-methylation of numerous soluble RNA-binding proteins (RBPs).

The increased methylation of chromatin-associated proteins and H3 following CDDP treatment was shown to induce the senescence-associated secretory phenotype (SASP) transcriptional program, which serves to counteract the apoptosis triggered by sustained genotoxic stress promoting senescence (Faget et al., 2019).

In addition, we were intrigued by the global hypo-methylation of RNA-binding proteins triggered by CDDP, which -we speculated- could promote their phase separation and stress granules formation, in light of published evidences indicating R-methylation as a major player in the regulation of RBP-RNA interaction and -in turn- the formation of transient, membrane-less organelles (MLOs), composed by RBPs and RNAs that aggregate as the result of liquid-liquid phase separation (LLPS) processes.

To investigate this new cellular mechanism, we coupled the Orthogonal Organic Phase Separation (OOPS) strategy (Queiroz et al., 2019) with quantitative proteomics and pharmacological inhibition of PRMTs to profile global changes of RBP-RNA interactions linked to protein R-methylation dynamics. The observation that the presence of subset of RBPs is reproducibly altered in the interface fraction enriched by OOPS upon treatment with PRMT type-I (but not type-II) inhibitor suggests that R mono-methylation and asymmetric di-methylation levels in these proteins modulate their interaction with RNAs. Moreover, we observed that cell treatment with the same PRMT type I inhibitor induces LLPS of some of these RBPs, whose interaction with RNA is modulated by the same treatment (Maniaci et al., 2021). Overall, our data provide first evidence that R-hypomethylation is a mechanism through which the cell subjected to CDDP-replicative stress alter the affinity to RNAs, subcellular localisation and MLO formation of candidate RBPs through the modulation of protein-R methylation and that this impacts gene expression post-transcriptionally, with effect on resistance to drugs.

AL22

An AI-driven leap forward in peptide identification through deconvolution of chimeric spectra

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Objective

Chimeric spectra are estimated to constitute >40% of DDA data, violating the assumption that one spectrum represents one peptide. Here, we describe a new intelligent search algorithm (CHIMERYS) 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

Our new algorithm uses accurate predictions of peptide fragment ion intensities and retention times provided by a 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. The algorithm 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. Searches can be triggered from laptops and conventional workstations (via a node in Proteome Discoverer 3.0 software) and are parallelized in the cloud.

Results

Analyzing a HeLa tryptic digest (1 hour gradient) with our new algorithm 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 our new algorithm 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.

Our new algorithm 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 outperforms other search engines (e.g. SequestHT) on data of different complexity such as body fluids and organisms from all kingdoms of life.

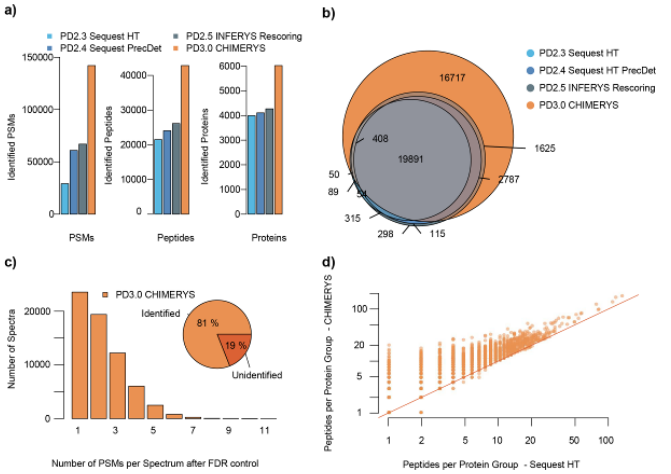
Conclusion

We present the first highly scalable, cloud-native, microservice-based and AI-powered search algorithm for the intensity-based deconvolution of chimeric spectra.

Figure legends

Figure 1. CHIMERYS doubles peptide identifications in single-shot full proteome DDA datasets. a) Number of PSMs, peptide and protein groups for a HeLa cell lysate. b) Overlap of peptide identifications between different search engines and processing workflows. c) Number of PSMs per spectrum and identification rate achieved by CHIMERYS demonstrates the extent of the chimeric spectra problem in DDA data. d) Number of peptides per protein group identified by CHIMERYS or Sequest HT demonstrates the increase in sequence coverage when using CHIMERYS.

Fig. 1



AL23

A proteogenomics framework to improve prokaryotic genome annotations

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Objectives: Address dilemma that accurate prediction of all protein coding genes is still an unsolved issue. Develop a broadly applicable proteogenomics solution that allows researchers to identify missed protein-coding open reading frames (ORFs) in prokaryotes. In particular, small proteins below ~100 amino acids, which carry out many important biological functions [1] are often missed.

Methods: We first check whether the genome of a reference strain is completely sequenced [2] and otherwise assemble it -or microbiome/clinical isolates- *de novo* [2-4]. To capture the entire protein coding potential, we hierarchically integrate reference genome annotations that can differ substantially, results from *ab initio* gene prediction algorithms and a modified six-frame translation of all possible ORFs that considers alternative start codons [4]. This integration is enabled by extending our PeptideClassifier solution [5], which allows to create a large but minimally redundant integrated proteogenomics search database (iPtgxDB) where ~95% of the peptides unambiguously identify one protein. We also create a general feature format (GFF) file that, when loaded in a genome browser, transparently visualizes all annotation differences and can be overlaid with experimental data.

Results: Our proteogenomics framework allows to identify novel small proteins, new start sites and expressed pseudogenes in prokaryotic genomes using tandem mass spectrometry data. Complete genomes sequences provide the optimal basis for functional genomics. Notably, a fragmented Illumina short-read based assembly can miss conserved core genes [3], while the genome of a closely related reference strain can differ and even miss essential and disease-relevant genes (or amino acid variations), as for the clinically relevant *Pseudomonas aeruginosa* MPAO1 [2]. We illustrate the succesful application to a clinical reference strain [2], to consortia [6] and to clinical isolates [7]. We host a public web server (https://iptgxdb.expasy.org) [4] where researchers can create iPtgxDBs and GFF files for their organisms of interest using e.g state of the art mass spectrometry data (e.g. top-down and PASEF workflows) and improved sample preparation and data analysis [8].

Conclusion: Several extensions to our framework further enhance its functionality and allow researchers to use Ribo-seq data to create smaller custom search databases and to leverage the value of tandem MS-based identification of small proteins.

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AL24

DIA-NN enables deep and accurate SILAC proteomics

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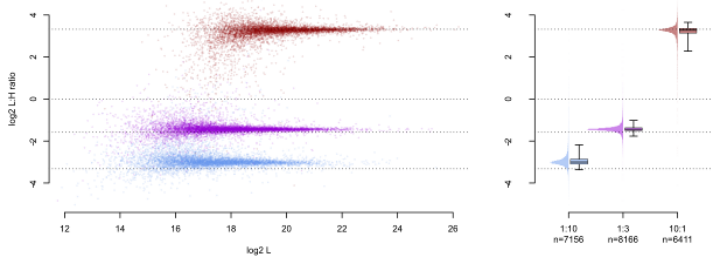
Stable isotope labelling with amino acids in culture (SILAC) is a powerful multiplexing technique for LC-MS proteomics and is an effective way of measuring protein turnover rates. However, when combined with data-dependent acquisition (DDA), SILAC substantially reduces the proteomic depth. Here, we implemented SILAC support in the plexDIA multiplexing module of DIA-NN, our universal neural network-based software suite for data-independent acquisition (DIA) data processing. Using a 125-min nanoLC method on a Q-Exactive HF-X, we show that SILAC-DIA achieves deep proteome coverage of ~8400 human proteins per injection, similarly to label-free DIA. We further demonstrate that multiplexing-optimised algorithms boost the accuracy and precision of SILAC quantities. Our solution paves the way for using internal SILAC standards to improve sensitivity as well as quantitative accuracy and allows protein turnover experiments to fully benefit from the advantages of DIA.

Fig.1. **Accuracy of light:heavy protein ratios reported by the SILAC module of DIA-NN.** Performance was assessed using light and heavy (K[+8] and R[+10]) HepG2 tryptic digests mixed in different proportions (1:10, 1:3, 1:1 and 10:1 L:H) and measured using a 125-min nanoLC method on a Q-Exactive HF-X in four replicates. Data were processed with DIA-NN in library-free mode. Protein ratios from 1:1 L:H runs were used to correct for the potential labelling or expression bias. Log2-transformed L:H protein ratios for 1:10, 1:3 and 10:1 mixtures were plotted against the respective intensities in the light channel. Boxes correspond to the interquartile range and whiskers to the 5%-95% quantile range.

Fig. 1



AL25

Limited but potentially functional translation of non-coding transcripts in HEK293T cells

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Question: Ribosome profiling provided evidence for the translation outside of canonical coding sequences such as short upstream open-reading frames (ORFs), long non-coding RNAs, ORFs in UTRs, overlapping ORFs and ORFs in alternative reading frames. This method, but also bioinformatics-based prediction and RNA-sequencing reported translation of thousands of ORFs derived from non-translated regions (NTRs). Although such ORFs gained increased attention over the years, their actual coding potential and hence possible functions remain debated as protein products of only a fraction of them were identified by mass spectrometry. Here, we introduced a new workflow to discover protein products of NTRs at a large scale.

Methods: We reduced the overall sample complexity (by enriching N-terminal peptides of cytosolic proteins as such peptides are ideal proxies for translation) and combined it with an extend search space (combining the sequences of UniProt proteins, UniProt isoforms and publicly available Ribo-seq data) reasoning that this increased our chances of identifying NTR proteins. Further, we introduced rigorous (meta-)data analysis workflows to cope with the increased complexity of the search space and to mine identified peptides. A stringent filtering approach was found essential to retain confident translational evidence at the peptide level for NTRs.

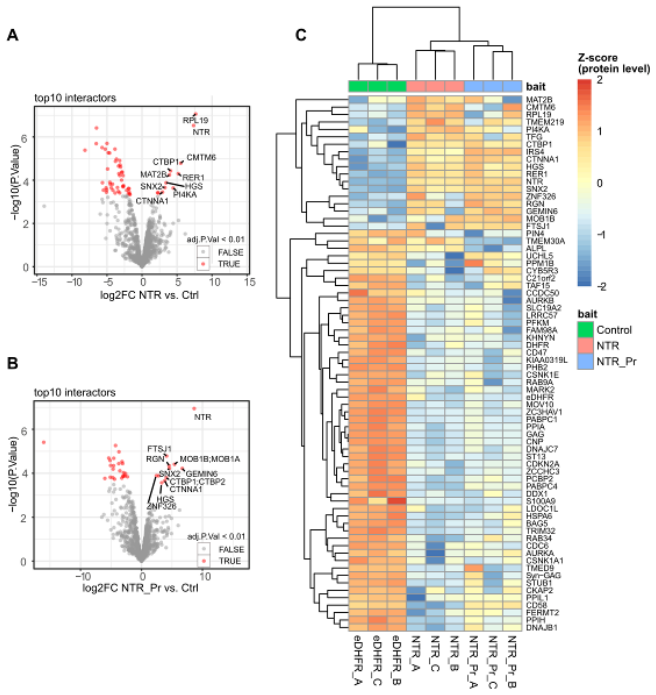
Results: We show that, theoretically, our strategy facilitates the detection of translation events of NTR transcripts as N-terminal peptides increase the chances

of identifying such peptides from NTR products about 7-fold. On the contrary, experimentally, we only identified 19 N-terminal peptides that possibly originate from NTR products (less than 1% of all identified N-termini). However, we detected N-terminal peptides pointing to other non-canonical translation events such as translation of 5"UTRs, and identified over 700 N-terminal proteoforms. The 19 NTR peptides were further curated by BlastP analysis, manual validation of their MS/MS spectra and inspection of sequencing data, leaving just 9 high-confident NTR originating peptides. Using Virotrap, we studied the interactome of one of these NTR proteins and identified several interaction partners pointing to the potential function of this novel protein (see Fig. 1).

Conclusions: Our stringent, evidence-based data analysis indicated that only a minority of NTRs gives rise to transcripts that are also translated to detectable, stable and potentially functional protein products. Thus, it is tempting to speculate that many ribosomal profiling studies overestimate the number of translated NTRs due to biases in sample preparation and data processing.

Fig. 1: Virotrap interactome analysis of a NTR protein (and N-terminal proteoform (NTR_Pr). A+B) Volcano plot showing the interactome of A) the NTR protein and B) its N-terminal proteoform. Significant proteins (FDR<0.01) are indicated in red. C) Heat map of all ANOVA significant proteins (FDR 0.01).

Fig. 1



AL26

Effects of space flight and bed rest on human muscle pathophysiology using single fiber proteomics

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Astronauts experience dramatic loss of muscle mass, decreased strength and insulin resistance, despite performing daily intense physical exercise that would lead to muscle growth on earth. With the deep space missions in horizon, understanding the molecular effects of unloading on muscle and coming up with countermeasures is crucial for the health of astronauts. Partially mimicking spaceflight, prolonged bed rest also causes muscle atrophy, loss of force and glucose intolerance. Slow muscle fibers, performing tasks of posture and tonic activity, and fast muscle fibers, engaged in phasic movements requiring more force, have been shown to respond differently to disuse. A fiber type-resolved proteomic approach to inactivity would thus be instrumental for further clarifying the molecular basis of muscle dysfunction affecting astronauts.

Protein from single muscle fibers were lysed and digested as described elsewhere (1) After method scouting on different instrument platforms, the final data were acquired using PASEF strategy. Digested and purified peptides were separated on an in-house packed column over a gradient time of 60 minutes using Thermo Easy nLC-1200. Eluting peptides were directly sprayed into a timsTOF Pro mass spectrometer via Captive Spray Ionization source. A DDA-PASEF method was used to acquire data and all raw data were processed using the MaxQuant computational platform

We quantified over 7300 proteins in the muscle fibers (2900 per single fiber on average) and 7100 in the whole muscle lysates of astronauts. We observed fiber type-specific changes which could not be extricated in

a whole muscle lysate. We then measured the muscle lysate of astronauts before and after a mission on the International Space Station with the same set up. From the integration of the two datasets, we could mine the contribution of specific fiber types to the phenotype of unloaded muscle.

Our study reveals proteomic signatures controlled by weight load in muscle and helps dissect the direct effects of unloading and disuse of skeletal muscle from the consequences of other complex challenges of spaceflight for human health

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AL27

Multiplexed Quantification of Nascent Proteomes with pPuro-TMT

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Dysregulation of translation has major implications in health and disease. Developing techniques to quantify changes in protein synthesis is of great value for biomedical research. A number of metabolic pulse labelling methods with heavy stable isotope-labelled and/or biorthogonal amino acids to quantify newly synthesized proteins (NSPs) using quantitative mass spectrometry have been developed. Key disadvantages of these existing methods are they require relatively long pulse times and special cell culture media.

More recently, O-propargyl-puromycin (OPP) has emerged as a metabolic label to enrich the nascent proteome. OPP is a puromycin analogue that is co-translationally incorporated into nascent peptide chains (NPCs), disrupting further elongation. OPP contains an alkyne side chain that can be pulled down via click chemistry on azide beads thus enriching for NPCs. Given the short pulse time and the fact that labelling requires no special media considerations, OPP is a promising addition to the expanding toolkit of methods to quantify translation.

Our aim is to combine OPP pulse labelling with tandem mass tag (TMT) - based chemical labelling (pPuro-TMT) to enable multiplexed quantification of protein translation. To adequately normalize differences in protein input across samples without obscuring global differences in translation, we use an OPP labelled spike-in reference. Our preliminary results show the effective capture of NPCs using OPP as a metabolic label. We believe that pPuro-TMT can become a powerful tool to study differences in protein translation across many conditions.

AL28

Deep Characterization of the High-Confidence Human Mitochondrial Proteome and its Dynamics

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Objective: Mitochondria are known as the cellular powerhouse. Moreover, they are interconnected with other cellular compartments and involved in numerous metabolic processes. Consequently, mitochondrial dysfunction is linked to numerous human diseases. To understand the involved molecular processes, detailed knowledge of the mitochondrial proteome is required. Human mitochondria are estimated to contain 1,100-1,500 proteins. However, ~25% of mitochondrial protein entries in common data repositories lack solid experimental evidence. Furthermore, we collectively counted 2,439 mitochondrial entries, which points to mitochondrial misassignments. Thus, we set out to establish and dissect the high-confidence human mitochondrial proteome.

Methods: We designed a multifaceted spatial proteomics approach consisting of (1) subtractive proteomics (2) importomics upon TOMM40 knockdown and (3) protein profiling across subcellular fractions obtained by differential and density-gradient centrifugation. Samples were fractionated by high pH RPLC and proteins were profiled by quantitative MS. Based on copy numbers per cell and iBAQ intensities measured in purified mitochondria, mitochondria-specific protein copy numbers were calculated. Using dynamic SILAC methods, we estimated mitochondria-specific protein half-lives. New mitochondrial proteins were analyzed by complexome profiling using BN-PAGE, interaction proteomics and loss-of-function methods.

Results: Our multifaceted spatial proteomics approach led to the identification of >10,000 proteins, with >8,000 proteins classified in mitochondrial fractions. Based on this data, we defined a human mitochondrial high-confidence proteome (MitoCoP) of 1,134 protein-coding genes, including 91 proteins previously not shown to be mitochondrial. Multiple cell line data confirm the consistency of MitoCoP across cell types. Comprehensive functional and disease-related classification of MitoCoP revealed that 460 MitoCoP genes are associated to one or more human disease phenotypes. We report mitochondria-specific copy numbers and half-lives for 1,016 and 832 MitoCoP components, respectively. MitoCoP dynamics spans three orders of magnitude, with protein half-lives from hours to months. Our data suggest that short-lived proteins represent regulatory checkpoints within central mitochondrial protein machines and biosynthetic pathways. Furthermore, based on MitoCoP complexome and interactome data, we identified so far unknown interactors of protein translocases and assembly factors of the oxidative phosphorylation system. Functional analyses confirmed small MitoCoP components as important factors for mitochondrial activity.

Conclusion: In sum, our data provide an unprecedented view on the human mitochondrial proteome, its organization and dynamics in a cellular context. MitoCoP will therefore be an important point of reference for better understanding functions and dysfunctions of mitochondria in human cells.

AL29

A Proteomics Approach for Studying Cross-Talk between Endothelial and Mesothelial Cells in a Co-Culture System

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Background

Peritoneal Dialysis (PD) is a life-saving renal replacement therapy. The composition of all currently available peritoneal dialysis fluids (PDF) triggers morphological and functional changes in the peritoneal membrane. Periodic exposure leads to vasculopathy, hypervascularization, and diabetes-like damage of vessels, eventually leading to failure of the technique. Cross talk between cells present in the peritoneal membrane modulate PD-associated changes. Currently, there is no in vitro system available to study processes relevant in these cells in close proximity. Here, we aimed to develop a co-culture model for investigating cell-to-cell communication by cellular proteome and secretome analyses.

Methods

For modelling the peritoneal membrane in vitro, mesothelial and primary endothelial cells were co-cultured in transwell plates. Cells were cultured under optimized conditions for simultaneous culturing in non-FCS starving conditions. Cells were treated with PD fluids in either co-culture or single culture conditions. The secretome was analyzed from the supernatant of the different cultures. An equalizer approach based on a combinatorial peptide ligand library was used to deplete high abundant proteins from the FCS. For quantitative proteomics of cellular and secreted protein abundances, isobaric labelling with a multiplexed liquid chromatography/mass spectrometry (LC-MS) approach was used.

Results

Analysis of the cellular proteome revealed perturbation of major cellular processes including regulation of cell death and cytoskeleton re-organization, which characterize PDF cytotoxicity. Co-cultured cells yielded differently regulated pathways following PDF exposure compared to individual cultures. Combined analysis of proteome and secretome showed different ligand-receptors pairs expressed uniquely under co-culture conditions regulating specific signaling pathways. Analysis of differentially expressed cellular and secreted proteins revealed variation in proteins related to angiogenesis, immune response, nitric oxide processes, and extracellular processes such as extracellular matrix reorganization, vesicle transport, and collagen deposition.

Conclusion

This study shows that harmful effects of PDF-stressed mesothelial cells also affect endothelial cells. Interestingly, both cells type react differently when co-cultured compared to the individual culture models, showing the importance of models that allow multiple cell type interaction to mimic the in vivo condition. In addition, we identified potential signaling axes

between the cell types that could explain pathophysiological changes of the peritoneal membrane during PD treatment. Characterization of PD-induced perturbations may allow identifying molecular mechanisms linking the peritoneal and cardiovascular context, offering therapeutic targets to reduce current limitations of PD and ultimately decreasing cardiovascular risk of dialysis patients.

AL32

Decoding the extracellular vesicle HLA-I peptide ligandome and interactome by mass spectrometry

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Objective: Extracellular vesicles (EVs) can modulate diverse processes ranging from metastasis to immunomodulation. With the possibility of tissue and immunological targeting, EVs are increasingly viewed as promising biopharmaceuticals to deliver drugs and vaccines. Although the total (phospho)protein content of EVs can now be routinely studied, the immunological and paracrine functions of EVs depend largely on the capacity of these particles to present antigens, and the interactome machinery on the EV surfaces to dock on recipient cells. Both remain highly challenging to study, given the low stoichiometry and heterogenous nature of EVs. Here we successfully applied AP-MS and XL-MS to study the immunopeptidome repertoire and interactome of EVs, ato provide key insights to guide EV-based biopharmaceutical development.

Methods: EVs were isolated by ultracentrifugation and the purity and quality of EV preparations was determined by multiple techniques (NTA, NS-TEM, Western blot, comparative proteomics). For HLA-I immunopeptidome characterization, 100 ug of EVs were immunocaptured with W6/32 antibody. HLA-I complexes were eluted with acid and HLA-I ligands were separated by 10 kDa filtration, desalted and analyzed by LC-MS/MS. Structural characterization of EVs was performed with an isogenic a pair of metastatic breast cancer cell lines. Intact EVs were cross-linked with 0.5 mM DSS in PBS, lysed and digested into peptides. Cross-linked peptides were enriched by SCX chromatography, desalted and analyzed by LC-MS/MS. Data was analyzed with pLink, at 1% FDR and only cross-links with ≥2 CSMs were retained.

Results: Our highly sensitive immunopeptidomics approach identified more than 3,400 HLA-I peptide ligands from 100 µg of EV material. Compared to cells, EVs over-represented HLA-B complexes and ligands, as well as cysteinylated ligands that may modulate immune responses. XL-MS of metastatic breast cancer extracellular vesicles detected active conformations of multiple protein complexes in EVs that have been linked to metastasis. Our cross-linking data corroborated strongly with structural predictions of previously uncharacterized regions of Moesin and indicated the existence of novel back-to-back conformations of Annexin A2, and also supported the existence a potent metastasis-inducing complex formed by αβ-integrin and CD151 in the EVs.

Conclusion: Here we demonstrate that affinity and crosslinking approaches coupled to the sensitivity of mass spectrometry can aid in functional and mechanistic studies of extracellular vesicles. These new applications can provide unique windows to accelerate future developments of EV-based biopharmaceuticals.

AL33

Chemical crosslinking combined with mass spectrometry for investigating the RNA/DNA–protein interactome

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Objective: A highly complex and dynamic network of proteins interacting with DNA and RNA molecules supports the viability of cells by driving processes of gene expression, genome replication and DNA repair.

Identifying the proteins involved, and elucidating how they interact with RNA and/or DNA, has long been a focus of research. In studies of RNA–protein complexes, mass spectrometry (MS) combined with UV crosslinking has been established for cell-wide studies, and this combined approach has made it possible to identify proteins and to pin-point the peptide regions involved, often down to amino-acid resolution.

However, in corresponding studies of DNA-binding proteins, crosslinking combined with MS has not been broadly applied as DNA crosslink purification and identification remains incomparably more difficult. In the work to be described, we show that chemical crosslinking overcomes certain limitations of UV crosslinking, e.g. preferential crosslinking of uridine/thymidine and it is applicable for studying both RNA–protein and DNA–protein interactions in a cell-wide manner.

Methods: We applied different chemical crosslinkers on isolated RNA- or DNA-protein complexes or on live cells. The samples were digested with nucleases and endoproteinases and enriched for crosslinked peptides carrying RNA or DNA (oligo)nucleotides. Enriched crosslinks were subjected to MS analysis and data were searched with a novel open-source nucleotide crosslink search engine, NuXL.

Results: Chemical crosslinking targets nucleotides and amino acids different from those targeted by UV crosslinking, and it thus extends the UV-crosslinking approach by yielding additional and complementary information about the RNA/DNA-interactome. A novel open-source nucleotide crosslink search engine, NuXL, offers crosslinking method (i.e. UV, chemical reagents) adapted search settings and allows for the assignment of RNA- and DNA-crosslinked proteins, including their crosslinked domains and amino acids.

Conclusion: We present a simple and straightforward chemical RNA/DNA-protein crosslinking MS workflow, that, combined with data analysis tool NuXL, enables to study DNA/RNA interactomes in live cells, providing complementary information to conventional UV crosslinking approaches.

AL34

Modulation of protein redox signaling abolishes resistance of leukemic cells to azacytidne

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Myelodysplastic syndromes (MDS) is clonal hematopoietic disorder characterized by ineffective blood cell differentiation and high risk of progression to acute myeloid leukaemia (AML). The treatment of MDS patients is directed at inhibiting DNA methylation with Azacytidine (AZA), which significantly prolongs patient survival. However, one third of patients are primarily resistant to AZA while the rest of patients develops AZA resistance. The molecular mechanisms underlying AZA resistance are yet to be understood, however our preliminary evidence pointed to a role of cellular redox (reduction/oxidation) homeostasis and protein thiol oxidative modifications in cellular response to AZA.

We herein applied a quantitative mass spectrometry-based proteomic approach to identify protein targets of oxidative modifications in a cellular MDS/AML model of AZA resistance that was previously developed in our lab. In three biological replicates we identified and quantified 7553 cysteine peptides of 2885 proteins. We showed that cells resistant to AZA (AZA-R) have distinct redox molecular landscape compared to AZA sensitive cells (AZA-S). Both AZA-S treated with AZA and AZA-R reach high protein oxidation levels pointing to the presence of oxidative stress in these cells. We identified 402 cysteines of 262 proteins to be significantly higher oxidized in AZA-R compared to AZA-S cells. The redox sensitive proteins of AZA-R cells mediate a variety of biological processes such as regulation of energy homeostasis, apoptosis or response to oxidative stress. We noted that AZA led to oxidation of several cysteines of protein redox sensor KEAP1, a regulator of NRF2 transcription factor that mediates antioxidant defence and that in AZA-R cells KEAP1 cysteines are constantly oxidized. We found out that by modulation of redox homeostasis via cysteine modification of KEAP1 AZA-R cells regain sensitivity to AZA.

Taken together, our data indicate that MDS/AML cellular response to AZA depend on cellular management of redox homeostasis. This notion further strengthens our broader hypothesis that global changes in redox environment influence cellular

metabolic adaptation which may also involve therapeutic resistance to AZA during clonal progression in MDS.

AL35

Profiling the cellular signalling state by targeted phoshoproteomics

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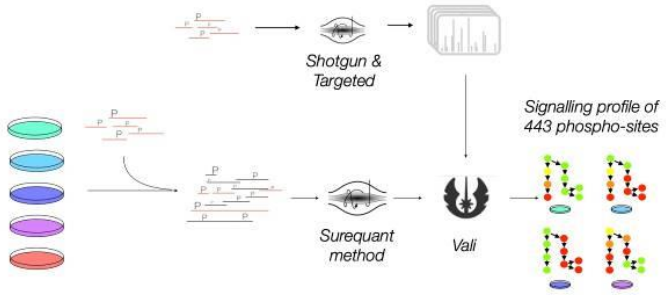
Normal cell functioning is regulated by a complex signalling network that responds to various extra- and intracellular signals like growth factors, cytokines and nutrients. Aberrations in cellular signalling often lead to disease, for example cancer. Understanding the functioning of the cellular signalling network, as well as changes in the network between healthy and diseased cells is a key challenge in biology research.

We have developed a targeted phospho-proteomic assay, that aims to provide a high-throughput, quantitative, reproducible profile of the cellular signalling state. The assay enables sensitive measurement of the phospho-sites that give a comprehensive overview of the cellular signalling state. The panel includes 443 phospho-peptides mapping to 249 proteins in all major signalling pathways. The phospho-peptides are measured in a targeted fashion, using internal standard triggered PRM (Surequant).

The data is analysed with a newly developed in-house tool: Vali. This tool enables semi automatic analysis of PRM data and is optimised to work directly with Picky generated spectral libraries. We confirmed sensitivity and accuracy of quantification of the PRM-Vali pipeline using a SILAC dilution series. We observed linearity in quantitation down to 63-127 fold ratios using the described setup in a pure automated fashion.

Performance of the assay was determined by measuring perturbations of HCT-116 colon cancer cells with growth factors and cytokines. We were able to validate previously identified signatures of these perturbations (for example, increased phosphorylation of Met Y1234 when treated with HGF, EGFR Y1172 when treated with EGF and Jun S63 and S73 when treated with TNFalpha). The assay will furthermore be applied to a colon cancer cell line panel to uncover previously unknown synergistic feedback mechanisms.

Fig. 1



AL36

On-bead *in vitro* kinase/phosphatase assays (OBIKA/OBIPHA) for proteome-wide target screens

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Introduction and question

The regulation of protein phosphorylation and dephosphorylation reactions by kinases and phosphatases, respectively, are essential for eukaryotic cell homeostasis. Dysregulation of underlying mechanisms may lead to severe consequences and is one of the main pathogenic factors of devastating diseases, including cancer [1]. Thus, major efforts are being invested in characterizing the relationships between kinases, phosphatases, and their substrates. Pharmacological or genetic *in vivo* phosphoproteome screens usually provide hundreds of potential targets. In combination with *in vitro* kinase/phosphatase assays of single proteins, direct targets can be discriminated from indirect effects [2, 3]. However, the low throughput of *in vitro* assays is a large limitation yielding only few *bona fide* target sites per study.

Methods

In this project, we developed highly multiplexed on-bead *in vitro* kinase/phosphatase assays (OBIKA/OBIPHA) using immobilized cell proteome under native conditions as input to screen for direct targets on a proteome scale.

Results

Combining *in vivo* and OBIKA analyses, we identified 187 sites on 157 proteins as direct, *bona fide* target sites of ULK1, which is a key regulator of autophagy [4].

Now, we aim at characterizing direct targets of various protein phosphatase 2A complexes.

Conclusions

Our methods significantly increase the throughput of *in vitro* screens of direct kinase/phosphatase targets, which will accelerate research of phosphorylation networks and aid in the development of specific kinase or phosphatase inhibitors.

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Fig. 1

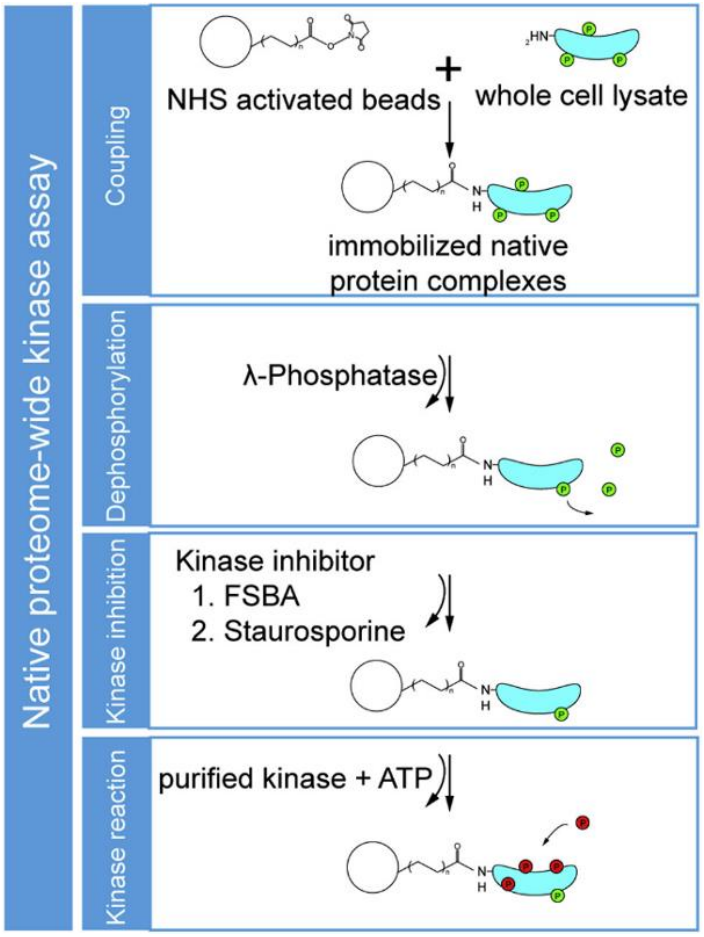
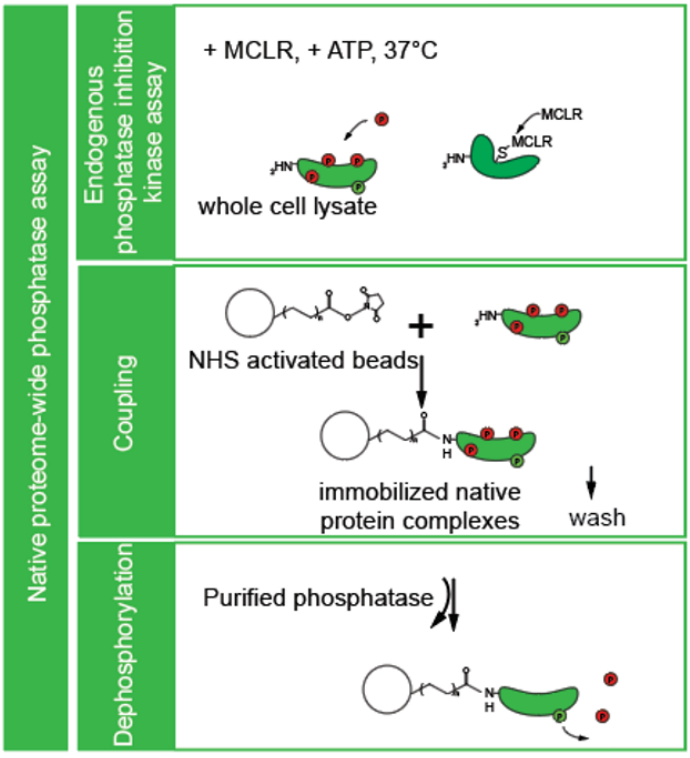


Fig. 2



AL37

Quantification of synuclein proteoforms in patient plasma

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Objective - Synucleins (alpha, beta, and gamma) are pre-synaptic markers implicated in neurodegenerative diseases (Mehra *et al.*, 2019). These proteins are cited as biomarker for Parkinson"s disease dementia (PDD), Dementia of Lewy body (DLB) and the Multi system atrophy (MSA) to alpha-synuclein and Alzheimer"s Disease (AD) and Creutzfeldt Jacob Disease (CJD) to beta and gamma synucleins (Oeckl *et al*., 2016). The quantification of these targets is clinically relevant and Mass spectrometry assay is the method of choice. Synucleins are subject to mutations, alternative splicing, and post-translational modifications. An antibody free method was developed and validated to quantify alpha, beta, and gamma synucleins proteoforms starting with 95 µl of plasma. Patient cohort of 155 samples was analyzed with 57% PD, 22% LBD, 6% MSA, and 15% of control (CTRL) patient samples.

Methods - From 95 µl of plasma, proteins were differentially precipitated. Synuclein enriched fraction was clean-up on AssayMap BRAVO (Agilent Technologies) and digest with trypsin/lys C. Liquid Chromatography-Multiple Reaction Monitoring (LC-MRM, Nexera Mikros – 8060, Shimadzu) method was developed to monitor 14 peptides originated from the alpha, beta and gamma synuclein sequence and one hemoglobin peptide. Method was validated following the EMA guidelines by studying LLOQ, linearity, inter and intra assay precision, matrix effect, sample stability before and after sample preparation, parallelism and dilution integrity. Data processing was performed with LabSolutions Insight (3.7). To the same samples, alpha synuclein was quantified with Meso Scale Discovery platform (U-plex human alpha synuclein kit). On the same cohort, 400 µl of CSF were used to quantify synuclein proteoforms with the same validated LC-MRM method.

Results - Quantitative assay based on LC-MRM was validated on total alpha (140aa), beta (134aa) and gamma (127aa) synuclein peptides, and 4 alpha synuclein proteoforms (126, 112, 98 and 41) using 3 proteotypic peptides (one for 126/98, one for 112/98 and one for 41). After cohort quantification, alpha and beta synucleins were quantified in plasma with good performances and are able to statistically discriminate CTRL vs PD; CTRL vs LBD; CTRL vs MSA, and MSA vs PD. A slight correlation was found between the quantities obtain between MSD and MS assays. No correlation was found between CSF and plasma fluids with the LC-MRM quantification.

Conclusion – MS based assay was successfully developed and validated to synuclein proteoforms. Analysis of patient cohort indicate the capability of this assay to discriminate PD, DLB and MSA with a specificity of 81% and a sensitivity of 87%. Immuno-precipitation of alpha and beta synuclein coupled to LC-PRM detection is currently in process of validation to quantify new synuclein"s proteoforms (truncations, PTMs).

AL38

Identification of proteomic profiles signifying the time-to-recurrence of glioblastoma multiforme patients

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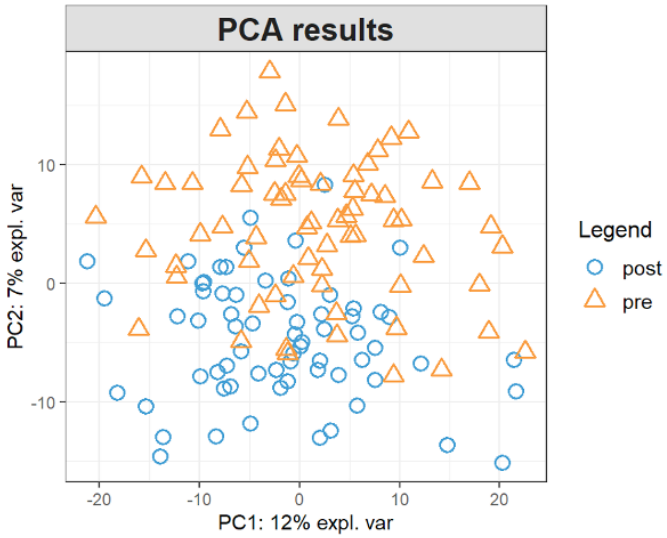
With 9 out of 10 patients developing recurrences after initial surgery and a resulting 5-year survival rate below 10%, treatment options for the most prevalent type of brain cancer - glioblastoma multiforme – urgently need to be improved. A main challenge are diagnostics of recurrent tumors via radiologic imaging, which are too insensitive to detect recurrences in early stages, and too costly and invasive to be used frequently. Estimations of patient individual times-to-recurrence (TTR) after initial resection could therefore enable more targeted checkup regimens and increase the chance for diagnosing recurrences in time for successful repeat surgery.

For our study, we have investigated serum from 60 patients before (pre OP) and after (post OP) initial surgery. Similar samples from recurrences were available from 8 of the same patients. Due to the dynamic range of serum protein expression, we depleted 14 highly abundant serum proteins. Using liquid-chromatography mass-spectrometry in data independent acquisition mode, we identified and quantified on average 812 protein groups in each sample. By comparing protein expression patterns from pre- and post OP samples, as well as from initial and repeat surgeries, we intend to detect proteomic profiles correlating to the TTR.

In a first general hierarchical clustering, a strong inter-patient heterogeneity of samples became apparent, accentuating highly individual serum proteome compositions that are more pronounced than proteome perturbations through surgery. Nevertheless, we observed differing proteomic patterns between pre- and post OP samples via principal component analysis and linear modeling. Differentially abundant proteins in post OP samples showed an inflammation fingerprint, including the elevation of 4 acute-phase proteins, which likely was a cause of the highly invasive surgery. Within hierarchically clustered pre OP samples, we could identify three biologically distinct patient subgroups. Of particular interest were proteins, whose expression diminished after surgery, as they might be used as potential tumor markers. In another approach, we will analyze our data via Cox proportional hazards model to identify proteins, whose expression correlates with the TTR distribution. Candidate proteins from both analyses will be followed up in subsequent studies involving matching tumor tissue as well as genomic and transcriptomic evaluation. Integration of this data with results from recurrent tumors might then provide further insight into the biology behind recurrences.

Overall, in our study we generated high-quality proteomic data reflecting patient"s conditions. In forthcoming analyses, we will test for proteomic patterns correlating with the patient's time-to-recurrence and generate a model to predict progression free survival. This preliminary analysis suggests the presence of a serum proteomic profile that might help to estimate the TTRs of glioblastoma multiforme patients.

Fig. 1



AL39

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

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Pancreatic ductal adenocarcinoma (PDAC) late diagnosis is primarily attributed to its asymptomatic progression combined with absence of any reliable screening markers. This leads to one of the deadliest cancer with a 5-years survival rate less than 10%[1].

Diagnosis is provided by endoscopy-guided fine-needle biopsy (EGFNB) only, which is invasive, risky and with a poor level of negative predictive value (NPV). Nevertheless, EGFNB remains the gold standard for diagnosing PDAC and enabling the right treatment for the patients.

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[2]. Proteomic analysis of the soluble proteins led to over 2500 identifications, which were subjected to subsequent statistical analysis. To build the following protein signature score (PSS), we used several resampling methods[3–6] at different steps of the analysis and an algorithm derived from microarray analysis techniques[7,8].

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.

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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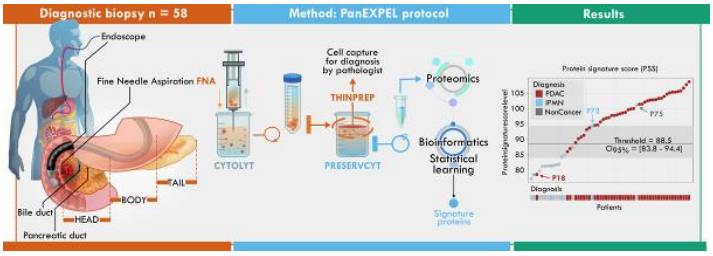
Fig. 1



AL41

Recent advances in proteotyping pathogens and environmental microorganisms by tandem mass spectrometry

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Question.

Tandem mass spectrometry-based proteotyping of microorganisms has key advantages over other approaches. Due to a greater number of signals recorded, the methodology allows the identification of microorganisms at highly resolved taxonomic levels and can manage complex samples (Grenga et al., 2019). Here, we document its application to pathogens and environmental microorganisms, test its sensitivity and explore the possibility of high-throughput for culturomics.

Methods.

Peptides generated with trypsin were analyzed by means of several high resolution instruments (LTQ-Orbitrap XL, Q-Exactive HF, Exploris480 mass spectrometers) operated in data-dependent acquisition mode. MS/MS spectra were identified using a cascade of searches conceived for maximizing metaproteomics results against a giant database comprising all sequenced genomes to date. The taxonomy of the microorganisms was established on the basis of the taxon-specific peptides and the taxon-assigned MS/MS spectra.

Results.

The distribution of assigned Taxon-Spectrum Matches according to the taxa present in the database was modeled, leading to the discovery of the "Phylopeptidomic" signature (Pible et al., 2020). This signature makes it possible to quantify the biomass of each microorganism present in a mixture. Sample preparation for proteotyping has been improved to analyze hundreds of isolates (Hayoun et al., 2019 & 2020), making this methodology truly amenable to culturomics. The proteotyping approach can be applied to clinical pathogens as well as poorly documented environmental microorganisms, whatever the branch of the Tree of Life. We further documented the methodology showing that the sensitivity of the method is 10E4 bacterial cells required for typing at the species level. Finally, a multiplexing methodology was developed to be able to analyze dozens of isolates per analytical run, and identify their potential antibiotic resistance at the same time.

Conclusions.

Overall, tandem mass spectrometry-based proteotyping is an interesting application of proteomics for rapid microbiological taxonomy. The taxonomic identification without a priori and the characterization of resistance to antibiotics pave the way for a new revolution in the clinical microbiology laboratory.

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Fig. 1

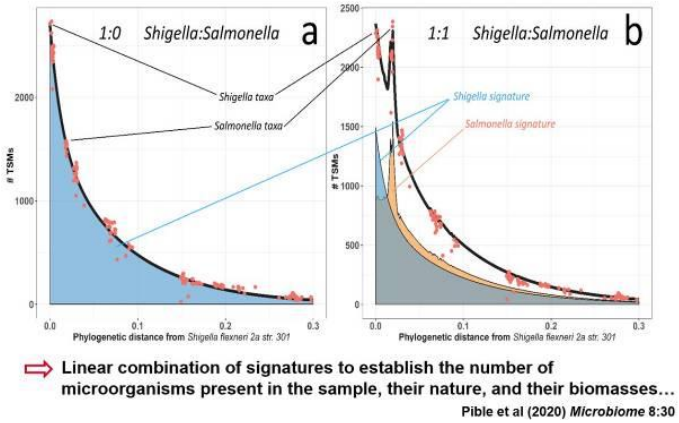
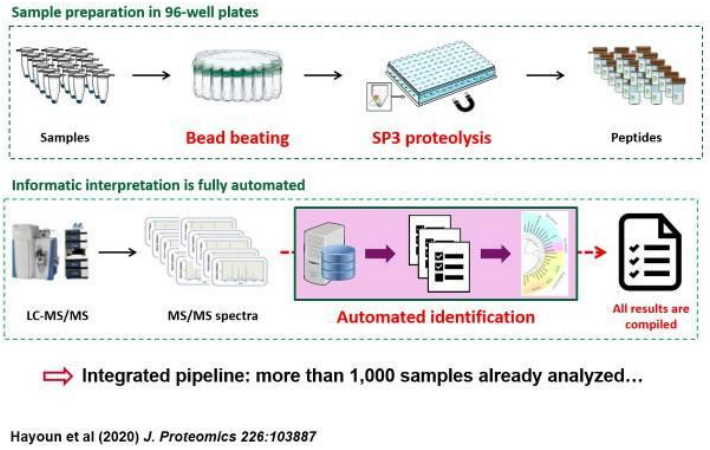


Fig. 2



AL42

Proteomics characterization of interferon-α subtype immune signatures during SARS-CoV-2 infection

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Objectives Type I interferons (IFN-I) exert pleiotropic biological effects during viral infections, balancing virus control versus immune-mediated pathologies and have been successfully employed for the treatment of viral diseases. Humans express twelve IFN-alpha (α) subtypes, which activate downstream signaling cascades and result in distinct patterns of immune responses and differential antiviral responses. Inborn errors in type I IFN immunity and the presence of anti-IFN autoantibodies account for very severe courses of COVID-19, therefore, early administration of type I IFNs may be protective against life-threatening disease. Here, we comprehensively analyzed the antiviral activity of IFNα subtypes against SARS-CoV-2 to identify the underlying immune signatures and explore their therapeutic potential.

Methods Primary human airway epithelial cells (hAEC) were prophylactically treated with different IFNα subtypes during SARS-CoV-2 infection. Functional characterization uncovered distinct functional classes of IFNs with high, intermediate and low antiviral activity. Four IFNs (α5, α7, α16 and λ3) representing the observed functional subclasses, were further studied using LC-MS/MS-based proteomics. We characterized the IFN-induced proteome profiles in hAEC from four donors at two time points before and during infection.

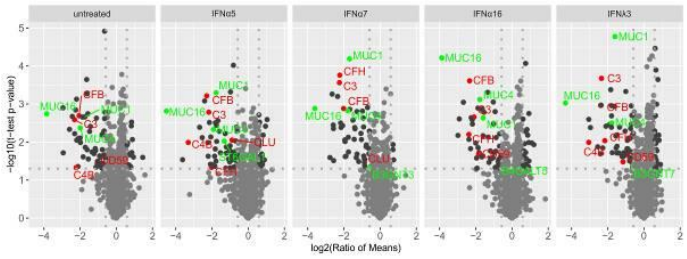
Results We uncovered distinct functional classes of IFNs with high, intermediate and low antiviral activity. In particular, IFNα5 showed superior prophylaxis against SARS-CoV-2 infection. We performed label-free proteomics to systematically assess the abundance of specific antiviral key effector molecules which are involved in type I IFN signaling pathways, negative regulation of viral processes and immune effector processes. Here, we describe the protein abundance patterns which are characteristic for IFN-specific antiviral activities towards SARS-CoV-2 infection. Furthermore, the analysis of two time points allowed us to monitor infection-related proteome dynamics. Interestingly, despite reduced viral replication in the presence of IFNs, we observed several mucins as well as complement factors consistently down-regulated as a consequence of SARS-CoV-2 infection.

Conclusions In this study we provide a global characterization of the antiviral response of different IFNα subtypes on various levels. We uncovered protein signatures which are able to significantly reduce SARS-CoV-2 infection and identified novel features after virus infection of primary cell types. Our study contributes to an enhanced understanding of the molecular landscape controlling SARS-CoV-2 infection and could thereby pave the way towards novel therapeutic approaches upon identification of key

cellular pathways and factors involved in SARS-CoV-2 infection.

Figure 1: Volcano plots of IFN-treated SARS-CoV-2-infected hAEC. Regulated proteins are colored according to their biological function: red = complement activation; green =O-glycan processing.

Fig. 1



AL43

Proteome analyses of *Bordetella pertussis* infection models as approaches to explore the re-emergence of whooping cough

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Question

Whooping cough caused by *Bordetella pertussis* (*Bp*) is regarded as re-emerging disease even in fully vaccinating countries. Besides vaccine inefficiency, intracellular survival of the pathogens leads to dissemination amongst the population (reviewed in [1]). Using global proteome approaches, we aim to elucidate bacterial as well as host responses in infection simulating models.

Methods

Bp Tohama I and an isogenic Δ*hfq* mutant were cultivated in iron-replete and iron-depleted Stainer-Scholte medium until late exponential growth phase. In addition, the wild type and an isogenic BP1092 mutant were cultivated in Stainer-Scholte medium with and without MgSO4. Bacterial proteomes were analysed in late exponential phase by nanoLC-MS/MS. In addition, human monocytic THP-1 cells were infected with *Bp* Tohama I (MOI 150) and after 2 h, non-internalized bacteria were killed with polymyxin B sulphate. The proteome of recovered intracellular *Bp* was examined 3 h and 48 h post infection using a gel- and label-free nanoLC-MS/MS approach. Data were compared to extracellular *Bp* grown under similar conditions. The response 16HBE14o- cells to an infection with the *Bp* wild type (MOI 1) was investigated by proteomics 8 h post infection.

Results

Upon internalization by human macrophages, 40% of the detected *Bp* proteins showed altered levels e.g., of proteins involved in stress response, iron uptake, metabolism, and virulence [2]. These data especially revealed a role of MgtC for *Bp* to adapt to the mildly acidic conditions inside phagosomes [3]. Also BP1092, a kinase of a two component system, was found increased in level after infection and relevant for the virulence of *Bp*. The chaperone Hfq, which was found relevant for bacterial survival inside in macrophages, modulated 33% of *Bp* proteins regulated during iron limitation *in vitro* [4]. On the other side, infected host cells altered protein levels involved in defence and membrane transport compared to non-infected control cells.

Conclusions

Our comprehensive proteome data of *Bp* infection models will help to understand its pathogenicity and paved the way for currently ongoing follow-up studies on immune responses to specific bacterial antigens.

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AL44

Engineered multi-nanoparticle panels enable unmatched depth and sensitivity in plasma proteomics

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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.

AL45

Development of a fully automated SP3 sample preparation protocol for high throughput proteomics on low amounts of starting material

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Objective

Over the past few years, a variety of innovative sample preparation solutions have been developed for high throughput proteomics. All those protocols aim at reducing the required starting material amount, the sample processing time, and the number of manual handling steps, which negatively impact reproducibility. In this context, there is still a room for developing fully automated workflows, especially on low protein amounts. Single Pot Solid-Phase enhanced Sample Preparation (SP3) has proven to be one of the promising alternatives to conventional techniques. We propose here the implementation and evaluation of a fully automated SP3 workflow developed on a liquid handling robot. This workflow decreases the time and workload for the analyst, offers increased reproducibility and precision for starting amounts as low as 1µg.

Methods

A range of total HeLa cell extracts using five different starting protein amounts from 1µg to 20µg was prepared with an optimized SP3 protocol on a liquid handling automated platform (Assay Map Bravo, Agilent). All peptide extracts were analyzed on a nanoLC-TimsTof Pro coupling (Bruker Daltonics) platform. Data were acquired in both Data Dependent Acquisition and Data Independent Acquisition Parallel Accumulation–Serial Fragmentation (DDA-PASEF and DIA-PASEF).

Results

More than 5000 proteins were identified for the conditions ranging from 2.5µg to 20µg starting amount with a standard deviation (SD) of less than 3%. More

than 4800 proteins could be quantified for 20µg and 10µg starting amount and more than 4200 of those were found in all of the six replicates.

More than 3900 proteins could be quantified for 5µg and 2.5µg conditions and more than 3400 of those were found in all replicates. Among those, the number of proteins having a Label-Free Quantification Intensity (LFQ-I) value with a Coefficient of variation (CV) less than 20% was found to be 3849, 3159, 3477, and 2700 belonging to 20µg, 10µg, 5µg, and 2.5µg conditions, respectively.

For 1µg starting amount, 3599 proteins were identified (SD 8%) and 2340 proteins could be quantified out of which 1644 proteins were quantified in all replicates. However, the number of proteins quantified with a CV on the LFQ-I values below 20% was limited to 458.

In DIA mode, the number of proteins sets identified were 7069, 7071, 7046, and 6943 for the conditions with 20µg, 10µg, 5µg, and 2.5µg starting amount respectively, with an SD of less than 0.6%. Out of those, 6362, 5297, 4954, and 4819 respectively were quantified with a CV below 20%.

Conclusion

Overall, the fully automated workflow demonstrates excellent reproducibility with a high number of proteins identified and quantified even for a low amount of starting material, down to 2,5µg. More than 3900 proteins can be reliably quantified using 4 hours of sample preparation time excluding overnight digestion, and 110 minutes of LC-MS/MS acquisition.

Fig. 1

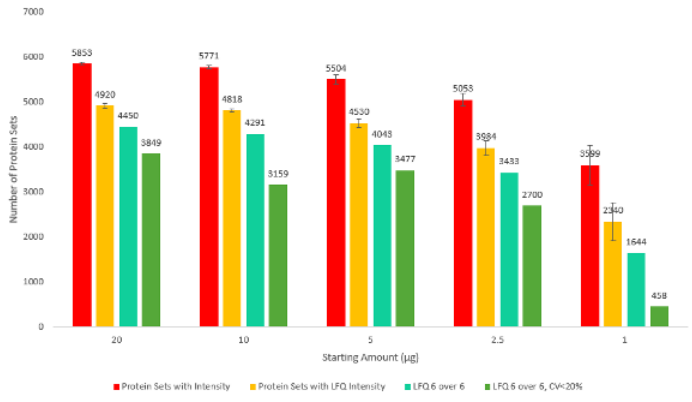
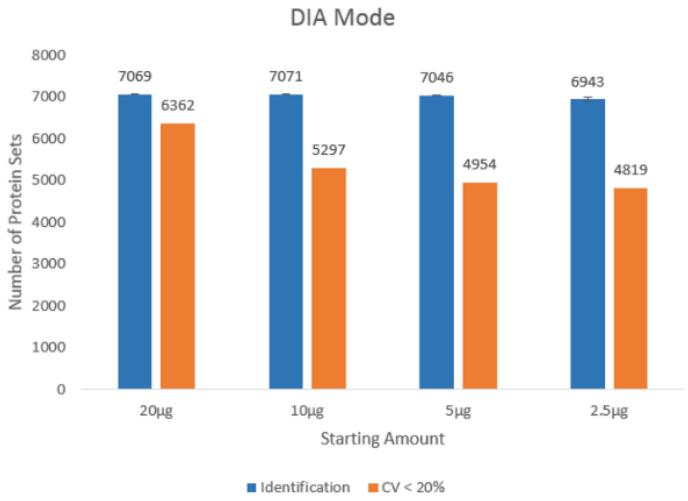


Fig. 2



AL46

A novel protocol for multiplexed or label-free single cell proteomics at unprecedented sensitivity

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Objective: Cellular heterogeneity is one of the main drivers of critical biological processes and to investigate these, single cell resolution is necessary. Recently global and hypothesis-free single cell proteomics (SCP) by mass spectrometry has complemented transcript-based techniques, yet low protein abundance impedes accurate and high-throughput quantification. Here we present the [proteoCHIP™] that in combination with the liquid- and cell-dispensing robot [cellenONE®] processes single cells in sub µL volumes, reducing adsorptive losses and chemical noise.

Methods: Single cells were sorted into 40-100nL or 300nL of master mix (0.2% DDM, 20ng/µL Trypsin, 100mM TEAB) for TMT or label-free samples, respectively, followed by incubation at 50°C 30min and at 37°C 4h or 50°C 2h. Multiplexed samples were labeled with 100nL 11-22mM TMT, subsequently quenched with 50nL 0.5% hydroxylamine 3% HCl and pooled via centrifugation. All samples were acquired

with the Waters nanoEase m/z BEH C18-column on an [Orbitrap Exploris™ 480] with [FAIMS Pro™].

Results: The [proteoCHIP™] allows to process up to 192 cells in parallel by multiplexing 12 sets of 16 cells (i.e. [TMTpro™]). All pipetting and dispensing steps are conducted within the temperature- and humidity-controlled [cellenONE®]. The samples are submerged in hexadecane to eliminate evaporation and guarantee constant concentration of chemicals. The oil solidifies at autosampler temperatures and does not interfere with chromatographic separation. After labeling, samples are pooled into the [proteoCHIP™]-lid via benchtop centrifugation, which is directly interfaced with the HPLC. The 2.5-fold master mix volume reduction limits adsorptive losses and improves enzymatic digestion resulting in 25% increased protein groups. Similarly, our label-free SCP workflow yields ~500 proteins per single HeLa cell. From only multiplexed single cells or in combination with a 20-times more abundant congruent carrier, we reproducibly identified ~900 or ~1100 protein groups per analytical run, respectively. Our efficient sample preparation workflow and loss-less sample acquisition resulted in unprecedented quantitative data quality with a median reporter ion signal-to-noise (S/N) of 255. This allowed for successful cell type specific clustering of 110 multiplexed HeLa and HEK cells. Furthermore, the analysis of *in-vitro* derived cardioids corroborated successful co-differentiation and cell specification of early cardiomyocyte and endothelial lineages.

Conclusion: The [cellenONE®] in combination with the [proteoCHIP™] allows for evaporation-free and loss-less processing of single cells via process miniaturization and direct HPLC injection. Our label-free workflow yields comparable protein identifications to state-of-the-art methods. Through eliminating the carrier channel our protocol outperformed reporter ion S/N of previous multiplexing SCP protocols by nearly 18-fold.

AL47

Unveiling drug resistance in FLT3 positive AML: a network-based approach

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Over the past years, tremendous scientific and technical progress enabled the development of new therapeutic strategies to kill tumor cells. Although chemotherapy and targeted therapy can significantly improve prognosis of cancer patients, a significant percentage of patients often develop drug resistance. Specific genetic alterations have been shown to confer primary resistance. Here we focus on internal tandem duplications (ITDs) occurring in the FLT3 gene in acute myeloid leukemia, a life-threatening, complex and heterogeneous disease caused by uncontrolled expansion of myeloid precursors. The different location of the ITD mutations in FLT3 has been recently associated to a distinct tyrosin kinase inhibitor (TKI) sensitivity and a diverse patient clinical outcome.

Here we profiled the TKI-dependent changes in the transcriptome, proteome and phosphoproteome of resistant and sensitive FLT3-ITD AML cells using deep sequencing and high-resolution mass spectrometry. This approach enabled us to quantify about 10,000 transcripts, 6,000 proteins and 16,000 phosphosites. To interpret these datasets, we developed a novel generally applicable strategy that enables to obtain predictive context-specific signaling models by overlaying transcriptomic and (phospho)proteomic datasets onto literature-derived causal network.

This approach suggested that TKI treatment differently affects CDK1 activity in resistant AML cells, impairing cell cycle progression.These hypotheses were validated in vivo and we also demonstrated that CDK1 inhibitory phosphorylation safeguards genomic integrity protecting FLT3-ITD cells from double strand breaks induced by commonly used therapeutic drugs. Here we show how the crosstalk between cell cycle control and DNA damage response represents a mechanism of primary resistance to both TKI and standard-of-care chemotherapeutic drugs. In conclusion, our deep proteomic analysis reveals both detailed and global mechanisms contributing to drug resistance in FLT3-ITD AML cells.

AL48

Proteogenomics for splicing variation and differential expression: a myotonic dystrophy type 1 mouse model study

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Question

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), 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.

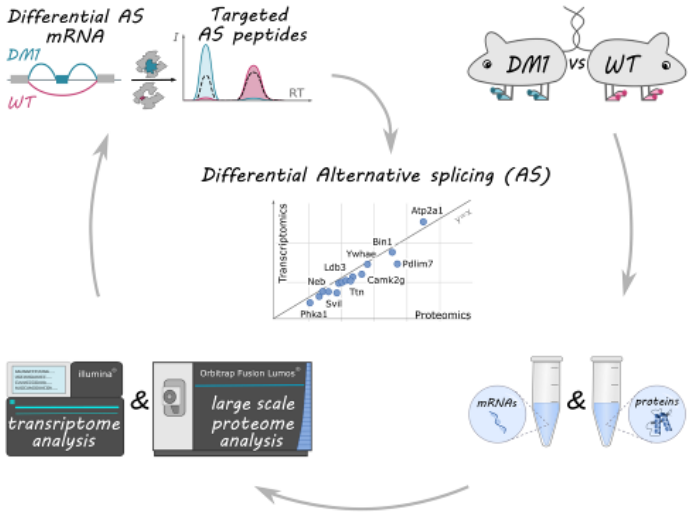
Results

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 for splice events assessed with targeted proteomics).

Conclusions

We believe that our work is suitable as a model for a robust and scalable integrative proteogenomic strategy defined by a staged approach of bulk transcriptomic and proteomic experiments informing targeted assays as a follow-on. Such a strategy advances our understanding of splicing-based disorders and helps establish robust splicing-specific disease biomarkers.

Fig. 1



AL49

Proteomic profiling of IDH-mutant gliomas enables prediction of chromosomal copy number variations

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Recurrent chromosomal copy number variations (CNV) are hallmarks of different types of brain tumors and status determination is an integral part of WHO classification. There is need for a better understanding of the consequences of gains or deletions involving whole chromosomal arms. A prominent example are IDH-mutant gliomas which are separated in two distinct types based on the deletion of chromosomal arms 1p and 19q. Oligodendrogliomas IDH-mutant are 1p/19q co-deleted while astrocytomas IDH-mutant are not. Therefore, determination of 1p/19q is important for prognosis and therapy. Upon demonstrating that the dda-PASEF based analysis of human formalin-fixed paraffin embedded (FFPE) tissue highly correlates with fresh frozen tissue we here present a workflow for in-depth proteomic profiling of IDH-mutant gliomas starting with small punches FFPE tissue. We've identified several novel potential protein biomarkers differentially regulated across two independent cohorts of oligodendroglioma and astrocytoma. Most strikingly, we were able to generate virtual copy number variation plots from the proteomic profile which we termed chromosomal protein ratio plots (CPRP). For CPRs we calculate the mean abundances of proteins grouped together by the chromosomal position of their coding genes and compared these to reference cohorts. Preliminary results from comparison of CNV plots from genome wide DNA methylation profiles and CPRs consistently displayed 1p/19q losses in oligodendrogliomas, as well as the majority of other whole chromosomal alterations present in individual tumors. This implies that gene dosage effects of whole chromosomal alterations are mirrored by the relative abundancy of numerous proteins. CPRP therefore, is a promising tool for the differentiation of tumors based on chromosomal copy number variation.

AL50

VCP inhibition affect protein homeostasis and deposit formation when inducing changes in proteomic patterns in the retina

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Mutations in the rhodopsin (RHO) gene are the most common cause of autosomal dominant retinitis pigmentosa (adRP), a group of inherited blinding disorders causing progressive photoreceptor degeneration in the retina. Dominant RHO mutations result in protein misfolding that leads to protein deposits. The deposits lead to proteotoxic stress and progressively impair photoreceptor survival. The ATP-driven chaperone valosin-containing protein (VCP) can detect protein misfolding in a yet unknown fashion, unfold as well as segregate such proteins and process the released polypeptides for proteasomal degradation. Counterintuitively to its role in protein quality control, pharmacological inhibition of VCP slows down photoreceptor cells death in animal models for adRP.

To identify the pathways by which VCP inhibition mediates photoreceptor survival, we performed comprehensive proteomic profiling without and after VCP inhibition of retinae from RhoP23H transgenic rats which carry a dominant RHO mutation in amino acid position 23. LC-MS-based proteomic profiling of retinal explants from these rats using a specific VCP inhibitor ML240 for 2, 6, and 48 hours uncovered alterations of the proteasome pathway. Furthermore, pathway and gene ontology analysis revealed that proteins associated with the signal transduction of light, oxidative stress response, and radical oxygen damage associated pathways changed between the three treatment timepoints. We detected different molecular signature profiles associated with treatment timepoints using clustering analysis. When analysed by western blot, Rho staining showed an increase of the major Rho band (mature form) in the ML240-treated retinae compared with vehicle-treated retinae. Western Blot analysis of markers indicating ER stress and unfolded protein response related stress (UPR) suggest alterations in proteostasis and energetic modulation after VCP inhibition.

Taken together, this analysis sheds light on the intricate higher-order interactions that are affected by VCP inhibition in degenerating photoreceptors and provide a resource for understanding the yet enigmatic function of VCP in protein quality control and cellular stress response.

AL52

Unravelling tissue-specific protein expression patterns: public data and machine learning joining forces

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Introduction

The first step towards thorough knowledge on the proteomic behaviour of tissue in a diseased state is the understanding of its proteomic composition in healthy circumstances. Possible downstream information such as biomarker and tissue leakage protein identification, and drug targets depend on the knowledge of healthy tissue-specific protein expression patterns. Using a myriad of experiments, from available public data, we trained a machine learning model to unravel these complex protein patterns on both tissue and cell type level.

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 a RandomForest model on protein abundances to classify samples in tissues and cell types. Subsequently, one-vs-all classification and the feature importance as F-scores are used to analyse the most discriminating protein abundances per class.

Results

With solely protein abundance, the model was able to predict tissues with 98% accuracy and cell types with 97% accuracy. Figure 1 and 2 illustrate the confusion matrices for tissue and cell type prediction respectively. We identified approximately 5 000 proteins crucial for classification, which accounts for 44% of the total amount of proteins present in the data. The F-scores describe a clear view of tissue-specific proteins and tissue-specific protein expression patterns. Application of the model onto diseased and cancerous samples show a drop in accuracy to approximately 67%.

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 post-translational modification data thus allowing even higher accuracy and understanding of modification patterns. Moreover, further in-depth analysis of the output from this model might lead to new biological insights.

References

[1] ionbot: a novel, innovative and sensitive machine learning approach to LC-MS/MS peptide identification. S. Degroeve et.al. bioRxiv 2021.07.02.450686

Fig. 1

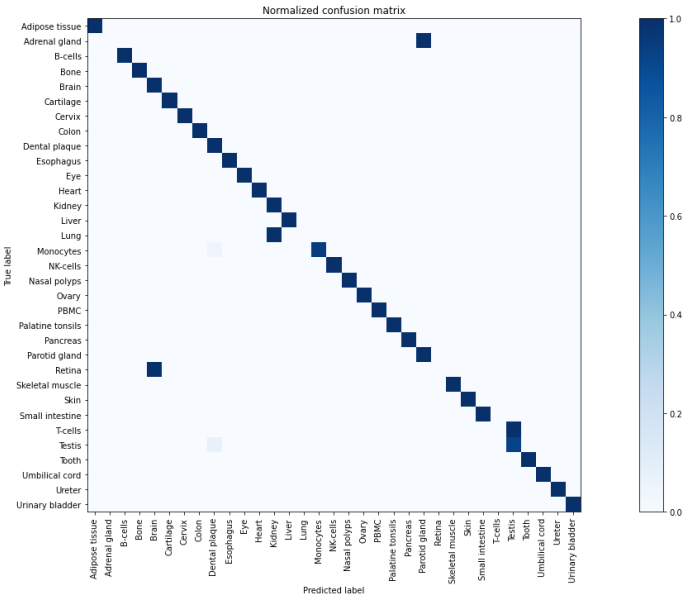
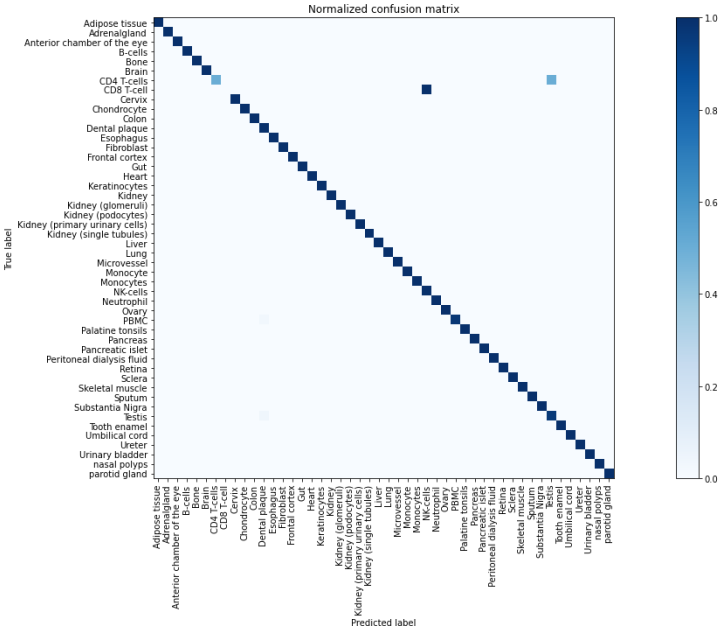


Fig. 2



AL53

ProteomicsDB: Towards a FAIR open-source resource for life-science research

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Introduction

Today, ProteomicsDB (https://www.ProteomicsDB.org) is a multi-omics and multi-organism platform for life science research. For this, ProteomicsDB has undergone many updates to cover additional data types and sources. However, systematic access to the data was limited to data from proteomics experiments, preventing access to a large section of the data and the development of alternative analysis and visualization strategies. Herein, we describe our recent efforts that focused on turning ProteomicsDB into a FAIR and open-source resource. We developed a new API to enable nearly full access to all stored data and started the re-implementation of the user interface, which will be available as an open-source project.

Methods

ProteomicsDB is based on the in-memory database infrastructure SAP HANA. Data is generally stored in RAM, this enables faster querying than a traditional database. The API of ProteomicsDB was redesigned following the OData standard. This enables an easier navigation between connected data and automatically generated documentation for each endpoint. Previously, the web interface of ProteomicsDB was implemented with a proprietary framework, SAPUI5. With the decision to move to the open-source vue.js framework, we will make all components open-source to allow re-used and addition by the scientific community.

Results

ProteomicsDB has turned into a multi-omics and multi-organism platform that enables data storage, visualization and analysis across different omics datasets, tissues and organisms. Over the last year, ProteomicsDB has grown to store protein expression values extracted from >36,000 LC-MS/MS runs covering more than 330 tissues of human origin, 128 tissues sampled from mice, 31 tissues from arabidopsis and 28 tissues from rice. We also extended the internal processing capabilities of ProteomicsDB to allow the re-scoring of datasets using Prosit, a deep neural network predicting the fragmentation characteristics of peptides.

In order to provide systematic access to nearly all data stored in ProteomicsDB, we redesigned the application programming interface (API). By this, any user or developer will gain access to the unified data model of ProteomicsDB and benefit from the seamless integration of the different levels of information. The API is supplemented with a new implementation of the user interface of ProteomicsDB using Vue. In combination, we believe that this will allow external developers to contribute novel tools and visualizations or the re-use of our components in in-house platforms. For example, the new spectrum viewer allows direct access to Prosit and the new protein feature viewer

allows the mapping of information gathered from proteomics experiments onto 3D protein structures.

Conclusion

We believe that these steps will allow us to turn ProteomicsDB into a FAIR resource, facilitating the findability, accessibility, interoperability, and reuse of the data and tools available in ProteomicsDB

AL54

PepGM: A probabilistic graphical model for taxonomic profiling of viral proteomes samples

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PepGM: A probabilistic graphical model for taxonomic profiling of viral proteomes samples

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Objective:

Inferring taxonomy in mass spectrometry-based shotgun proteomics 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. The goal of our newly developed method PepGM is to accurately identify viral strains from proteomics experiments using a probabilistic graphical model.

Methods:

For protein inference, any 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 present species adds an additional level of complexity. Current approaches rely on strategies such as peptide-spectrum-match counting or the use of unique peptides.

In PepGM, 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. The posterior probabilities of the taxa are computed through loopy belief propagation. PepGM is implemented in Python.

Results:

We show the propagation of peptide scores to taxa using a message passing algorithm and the results of taxonomic identification with a corresponding statistically sound score. We present the evaluation of using PepGM using selected viral mass spectrometric data sets (e.g., from SARS-CoV2 samples). In comparison to existing taxonomic annotation methods, PepGM reaches a high taxonomic resolution at the species level. In addition, we give an outlook on applications for functional protein profiling (e.g., annotations based on Gene Ontology or KEGG pathways).

Conclusion:

PepGM presents a robust probabilistic method for taxonomic profiling of viral proteome samples. It supports the statistically sound inference of taxa and eliminates the need for error prone heuristics.

Fig. 1

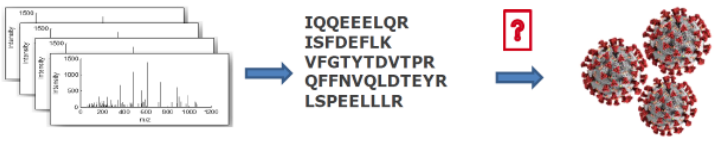
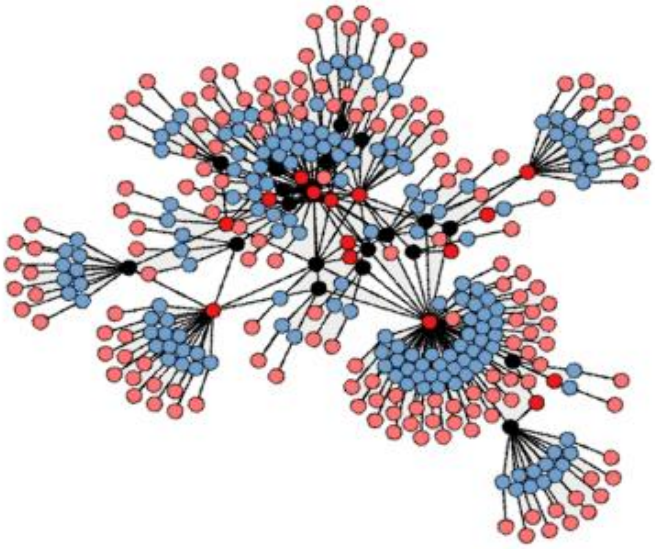


Fig. 2



AL55

Prosit-TMT: Deep learning model boosts identification of TMT-labeled peptides

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Introduction

Predicting peptides' fragment intensities and retention time has gained significant attention over the last few years. However, most studies focused on unlabeled peptides, although TMT-labelling is becoming a standard workflow covering single-cell and high-throughput proteomics. Particularly for TMT, increasing the number of confidently identified spectra is paramount as it provides identification and quantification information. Here we present Prosit-TMT, a novel deep learning model that accurately predicts retention time and fragment ion intensities for different combinations of fragmentation methods and mass analyzers. Furthermore, we show that this single model can re-score various TMT-based experiments resulting in up to 50% more peptide identifications for MS3-based acquisition methods.

Methods

The previously published Prosit architecture was modified to support TMT-labeled peptides. Briefly, an additional one-hot encoded input indicating the fragmentation method was added. Peptides used for training were labelled using TMT6plex. HCD and CID fragmentation was used for data acquisition, covering six different collision energies for HCD fragmentation. The model was trained with tryptic and non-tryptic peptides.

Results

A single model (Prosit-TMT) covering HCD and CID fragmentation was trained on 10 million and tested on 1.25 million spectra of TMT-labeled peptides. Prosit-TMT achieved very high levels of agreement between predicted and experimentally acquired spectra, reaching a normalized spectral contrast angle (SA) of 0.87 for CID and 0.92 for HCD. In addition, a model predicting the indexed retention time of TMT-labeled peptides was trained and tested on >600k, and >200k labelled peptides, showing a very high precision of Δt95%=79s.

To show Prosit-TMT's ability to re-score different datasets, we re-analyzed a TMT11plex human-yeast spike-in dataset acquired using HCD and CID fragmentation, each with Iontrap (IT) and Orbitrap (OT) readout. The number of confidently identified peptides was increased by ~12% for OT readouts and ~16% for IT readouts.

The results encouraged us to explore the effects on MS3-based acquisition methods. For this, we re-analyzed a TMT11plex cell line dataset that was acquired using MS2 and MS3 (HCD/IT for identification). We observed a substantial gain in peptide identifications for MS3 of >11k (13%) peptides vs>3k (4.5%) peptides in MS2. With re-scoring, MS3 substantially outperforms MS2-based methods. Earlier, we observed that spectra generated from low intense precursor peptides benefit most from re-scoring. To exploit this, we re-scored a TMT10plex MS3 (CID/IT for identification) dataset of human milk sampled every 12h for the first five days of lactation. Re-scoring increased the number of PSMs by 9924 (60%), peptides by 2282 (62%), and proteins by 614 (50%). Subsequent analysis revealed two clusters of anti-phase proteins that show an abundance behaviour connected to the day-night cycles.

AL56

Extensive Profiling of Direct Host-Virus Protein Interaction Contacts from Productively Infected Intact Cells

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Objective: Protein-protein interactions (PPIs) facilitate essential processes during viral infections. Accordingly, the global discovery of PPIs is important to understand the complex nature of host-virus relationships. Despite this importance, no method exists to identify host-virus interactions from virus-infected intact cells in a system-wide manner. Here we present Structural Host Virus Interactome Profiling (SHVIP) that combines *in vivo* cross-linking mass-spectrometry (XL-MS) of virus-infected cells and bio-orthogonal labeling during productive viral infection. SHVIP provides direct PPIs and their interaction contacts from native cellular environment. Importantly, taking advantage of selective bio-orthogonal labeling of viral proteins, it significantly improves sensitivity in detection of cross-links involving viral proteins.

Method: We established SHVIP using infection with Herpes-simplex virus type 1 (HSV-1). HSV-1 infected primary lung fibroblasts were labeled with bio-orthogonal amino acids L-homopropargylglycine (HPG) during productive viral infection. At 24 hours post infection, cross-linking was performed on intact infected cells using membrane permeable cross-linker disuccinimidyl sulfoxide (DSSO). HPG-labeled proteins (primarily viral proteins) were enriched by picolyl-azide agarose beads using a copper-catalyzed click-chemistry reaction. After standard proteolytic digestion, cross-links were enriched using strong cation exchange chromatography and subjected to LC-MS analysis for cross-link identification.

Result: We provided an XL-based PPI network consisting of ~350 PPIs based on ~4,400 cross-links, which covered ~50 % of the canonical HSV-1 proteins and provided many known and novel interactions. Interestingly, we found viral membrane proteins, tegument proteins as well as non-structural proteins cluster in different modules in the network dependent on their subcellular location. To further interrogate the XL-based interactions, we performed affinity-purification mass-spectrometry (AP-MS) experiments against HA-tagged viral proteins, including VP22 and alkaline nuclease UL12. While AP-MS data confirmed some interactions, we found several proteins that were not or only modestly enriched using AP-MS, suggesting context-dependent, low-affinity or modification-dependent interactions. Based on the interaction contacts from XL-MS, we mutated binding motifs for 14-3-3 proteins on the alkaline nuclease UL12 and an RVXF-motif for interaction with PP1 on VP22. Importantly, alanine substitution of the RVXF-motif dramatically impaired viral growth and production of viral proteins from all kinetic classes.

Conclusion: Our data represents the first extensive interactome from intact cells under productive viral infection. The resolved interaction contacts are crucial for the detailed understanding of host-virus interaction interfaces. In addition, the hereby developed SHVIP method is easily applicable to other viruses and cellular systems.

AL57

Development and utilization of a highly specific and sensitive multiplex serological multi-disease assay

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Objective

The COVID-19 pandemic imposed an immense and immediate need for accurate, sensitive and high-throughput serological assays, needed for both overarching epidemiological studies and evaluating vaccines. Here, the development and thorough validation of a high-throughput multiplex bead-based serological assay is presented together with an extensive utilization with 200.000 samples analyzed. Furthermore, a subsequent phase focusing on a pandemic laboratory preparedness with a multiplex and multi-disease antigen design and production is being implemented and thereby enabling future broad seroprevalence studies.

Methods

More than 100 representations of SARS-CoV-2 proteins were designed and included for initial evaluation, including antigens produced in bacterial and mammalian hosts as well as synthetic peptides. The five best-performing antigens, three representing the

spike glycoprotein and two representing the nucleocapsid protein, were further evaluated for detection of IgG antibodies in samples from 331 COVID-19 patients and convalescents, and in 2090 negative controls sampled before 2020. A long range of new variants of concern and variants of interest of the spike protein have subsequently been incorporated and evaluated.

Results

The finally selected antigens, represented by a soluble trimeric form and the S1-domain of the spike glycoprotein as well as by the C-terminal domain of the nucleocapsid. The sensitivity for these three antigens individually was found to be 99.7%, 99.1% and 99.7%, and the specificity was found to be 98.1%, 98.7% and 95.7%. The best assay performance was although achieved when utilizing two antigens in combination, enabling a sensitivity of up to 99.7% combined with a specificity of 100%. Requiring any two of the three antigens resulted in a sensitivity of 99.7% and a specificity of 99.4%. See Hober et al 2021: *https://doi.org/10.1002/cti2.1312*

The finalized serology assay has been used extensively for a long range of studies, see collection of published papers
here *https://covid19dataportal.se/data_types/health_data/serology-statistics/*

Examples will be discussed, with a focus on longitudinal analysis of patients and health care workers connecting serology to short and long term as well as mild and severe symptoms and also vaccine response over time. To complement the serology, we are also investigating the association of proteomic sized autoantibody profiles with long-covid.

Conclusion

These observations demonstrate that a serological test based on a combination of several SARS-CoV-2 antigens enables a highly specific and sensitive multiplex serological COVID-19 assay which is an important aspect of studying the effect of infections and vaccinations.

AL58

Spatial, Quantitative and Functional Deconstruction of Protein Interactions inside Cytomegalovirus Particles

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Question: Productive viral infections involve the packaging of the viral genetic material. The resulting particle gains the potential to infect new cells and start viral gene expression based on proteins that are co-packaged. Knowledge about virion composition and structure is therefore critical to understand viral pathogenesis. Herpesviruses assemble particularly large and complex enveloped particles that are difficult to characterize structurally due to their size, fragility and complex packaged proteome with partially amorphous nature. Here, using cross-linking mass spectrometry (XL-MS), we derived a structural interactome of intact extracellular virions of the betaherpesvirus Human Cytomegalovirus (HCMV). Methods: Extracellular HCMV particles were harvested from infected cell culture supernatant and cross-linked using disuccinimidyl sulfoxide (DSSO). The cross-linked HCMV particles were further purified using glycerol-tartrate density gradients, digested using a standard protein digestion protocol, and prepared for LC-MS analysis for cross-link identification. Results: Using the XL-MS method, we revealed a spatially resolved interaction map of 82 host and 36 viral proteins based on ~4,300 crosslinks. Using well-established viral proteins as localization markers, we categorized 36 viral proteins into four spatially resolved layers of the HCMV virion (i.e., capsid, inner tegument, outer tegument and glycoproteins). As an interesting observation, we found that the viral protein pp150 cross-links to proteins from all four layers in a domain-specific manner, indicating this protein to be spatially arranged across the entire virion. Furthermore, we found that 82 host proteins directly cross-link to viral proteins and that they are incorporated into viral particles in a layer-specific manner. To obtain a more comprehensive picture of the proteome landscape, we also obtained the copy numbers of the viral and the co-packaged host proteins using quantitative proteomics. We found that several host proteins are constitutive components that are recruited via specific host-virus interactions. Analyses with viral mutants showed that incorporation of the host phosphatase PP1 and 14-3-3 proteins is mediated by interacting with short linear motifs in the viral protein pp150. PP1 recruitment to HCMV particles

influences phosphorylation status inside virions, facilitates the start of viral gene expression and is important for efficient viral replication. Further, at late stage of infection, phosphatase activity regulates the interaction of 14-3-3 proteins with pp150. Conclusions: Our data gives a proteomic picture of intact HCMV particles in quantitative and spatial dimensions. It provides an organizational hierarchy of viral and host proteins inside the particle, which lays the groundwork for the structural understanding of crucial host-virus interactions.

AL59

Targeted proteomics as a tool to detect SARS-CoV-2 proteins in clinical specimens

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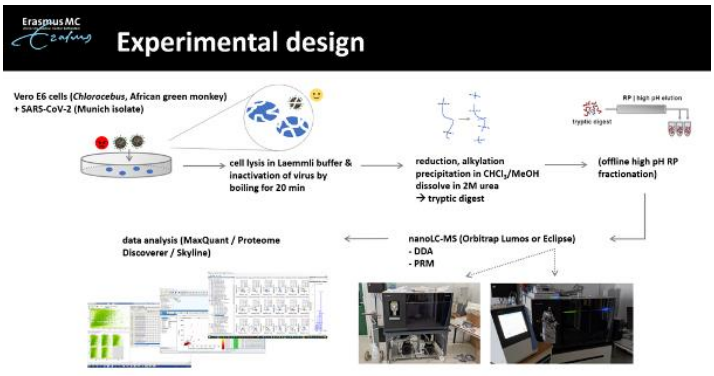
The rapid, sensitive and specific detection of SARS-CoV-2 and yet unknown viruses is critical in responding to the current COVID-19 pandemic and future outbreaks. In this proof-of-concept study, we explore the potential of targeted mass spectrometry (MS) based proteomics for the detection of SARS-CoV-2 proteins in both research samples and clinical specimens. We show that viral proteins can be detected and quantified in patient samples such as sputum and nasopharyngeal swabs with fairly high sensitivity, clearing the way for exploration of the use of proteomics technology in clinical and diagnostics labs. In addition, we present alternative sample preparation procedures to further optimize both the sensitivity of the assay and the LC-MS throughput.

The limit of detection for SARS-CoV-2 proteins in infected Vero E6 cells was assessed by parallel reaction monitoring (PRM) mass spectrometry on an Orbitrap Eclipse Tribrid mass spectrometer. SARS-CoV-2 was propagated on Vero E6 cells and infected cells were lysed and boiled to inactivate the virus. Proteins in patient nasopharyngeal and throat swabs or sputum samples in transport medium were first precipitated with acetone-TCA to remove excessive albumin. Protein pellet were then resuspended in Tris/HCl buffer and digested using the SP3 protocol. A selected set of unique SARS-CoV-2 Nucleocapsid and Spike tryptic peptides were subjected to a parallel reaction monitoring (PRM) mass spectrometry regime for targeted detection and quantitation.

Using PRM MS, the limit of detection for several tryptic peptides of the most abundant SARS-CoV-2 protein, Nucleocapsid, in a complex matrix background was estimated to be in the mid-attomole range (9E-13 g). Heavy labeled AQUA peptides were used to confirm fragment ion chromatograms and retention times of target peptides. Next, targeted MS was applied to the detection of viral proteins in various COVID-19 patient clinical specimens, such as sputum and throat and nasopharyngeal swabs. In these generally heterogeneous and often heavily contaminated samples, SARS-CoV-2 proteins could be detected with high sensitivity in all specimens with reported PCR Ct values of <24 and also in several samples with higher CT values. A clear relationship was observed between summed fragment ion chromatogram areas under the curve (AUCs) for SARS-CoV-2 tryptic peptides and Ct values reflecting the abundance of viral RNA. Subsequent steps for optimization of the procedure involve the improvement of quality and speed of sample preparation and LC-MS throughput. Using offline peptide fractionation, the sensitivity could be increased several fold, albeit at the expense of analysis speed. Taken together, these results suggest that targeted MS based proteomics may have the potential to be used as an additional, orthogonal tool in COVID-19 diagnostics.

Targeted protein mass spectrometry has the potential to be used as an additional tool in COVID-19 clinical research and diagnostics.

Fig. 1



AL60

SARS-CoV-2 natural immunity to vaccine-induced immunity: Systematic Evaluation of Humoral Response by functional proteomics

M. Fuentes[1]

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An unresolved question is to find out whether the mixed immune model (natural infection plus vaccine administration) can be a reproducible method to boost immunity. Here, we present a comprehensive and systematic analysis of humoral response against SARS-CoV-2 antigens in recovering COVID19 patients and Vaccine induced immune response based on functional proteomic approaches. Immunoglobulin profile was assed against SARS-CoV-2 virus in a multiplex assay including the main structural proteins, accessory and non-structural proteins by full-length structure and epitopes peptides. Additionally, two complementary multiplex platforms to characterize acute phase reactant and autoantibody profiles to further investigate the humoral response generated by the SARS-CoV-2 infection. The elucidation of common hallmarks of immunogenic epitope production in vaccinated and naturally protected individuals can bring valuable insights to reveal the immune processes underlying favorable disease progression. Our results can predict the increase in the intensity and breadth of the antibody response after vaccination in people previously infected with SARS-CoV-2

Fig. 1

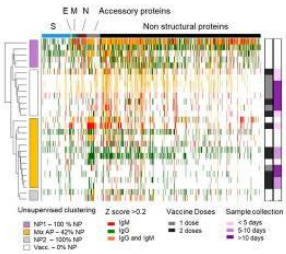
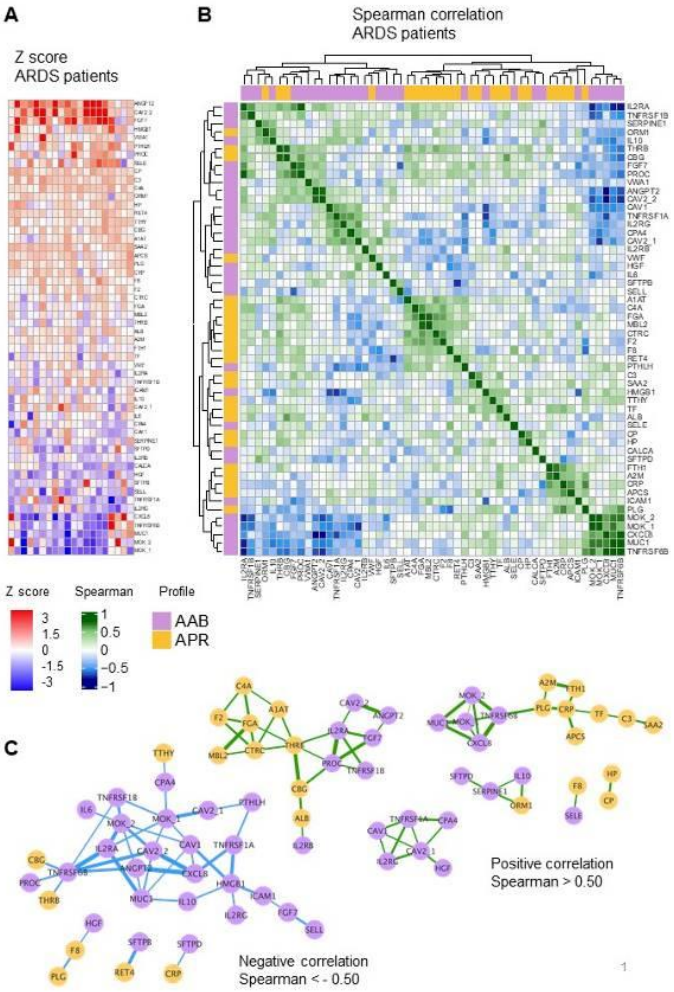


Figure 1

Fig. 2



AL61

Comprehensive proteomic mapping of human Amyotrophic Lateral Sclerosis

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Introduction: Amyotrophic lateral sclerosis (ALS) is a multifactorial neurodegenerative disease characterized by the death of upper and lower motorneurons. Despite considerable research efforts, the exact mechanism underlying the ALS pathogenesis is not yet fully understood. A complex interaction of genetic and environmental factors contributes to motorneurons damage. Redox dysfunction has long been associated with ALS, recent evidence points out that strong redox dyregulation associated to mitochondrial dysfunction (MD) may act as actual driver for motorneuron degeneration In addition mutations of several genes (e.g. *SOD1, TARDBP, FUS, C9orf72*) have been associated with ALS, however familial ALS (fALS) accounts for about 10% of all cases, while the remaining 90% of cases are sporadic ALS (sALS). Both forms, familiar and sporadic, are clinically indistinguishable with the same phenotype.

Question: In this perspective, this study aims at an in-depth characterization of ALS proteome in order to highlight common and different molecular features depending on gene variants mainly by looking at the reshaping of the redox profile.

Methods: To achieve this purpose, fibroblasts derived from human skin biopsies deposited in the AISLA ALS National Biobank at Centro Nemo, at Fondazione Policlinico A. Gemelli, Rome, Italy, have been used. In particular, fibroblasts from patients with both sALS (n=10) and fALS (including patients with *SOD1* (n=10), *TARDBP* (n=10), *FUS* (n=10), *C9orf72* (n=10) variants), and healthy subjects (n=10) have been selected. Label free proteomic analysis has been performed using HR-DDA approach on Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Scientific). In addition, mapping of persulfidation and Phosphorylation sites have been developed. Peptide identification and quantification have been performed by Peaks and MaxQuant softwares respectively; bioinformatics analysis was performed by Ingenuity Pathway analysis (Qiagen-IPA)

Results: Significant protein hallmarks and a metabolomic reshaping have been highlighted by making comparisons between ALS and controls, as well as within the ALS group. Particular attention has been given to the expression of proteins such as FUS, SOD1 and TDP43 that play a key role in ALS onset and progression. In-depth redox investigation shed light on the proteome imbalance towards the oxidation of protein thiol oxidation and persulfides, especially in SOD1-fALS

Conclusions: This investigation provides a comprehensive characterization of the redox landscape of ALS revealing molecular features hitherto unexplored.

AL63

Clinical Mass Spectrometry Center Munich (CLINSPECT-M): adding a proteomic component to molecular tumor boards

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Question: 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, Chimerys 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.

AL64

Precision proteomics in the general populatuion

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Precision medicine aims to improve patient care by improving treatment possibilities based on genetics, molecular phenotypes, environmental, and lifestyle factors. However, applications are often limited to treating those with a diagnosis or severe condition, thus preventing diseases in the general population is still not within reach. To fully unlock the capabilities offered by recent proteomics technologies to analyse circulating proteins [1,2], and to include a wider range of human conditions, new analytical capabilities linked to patient-centric sampling at home, such as dried blood spots (DBS), will be an essential cornerstone [3].

We successfully utilised advanced and precise sampling methods distributed by regular mail to monitor the effect of COVID-19 in general populations and developed analytical routines using multiplexed serology [4] and proteomics approaches [5]. The presentation will present insights and findings from a variety of ongoing COVID-19 projects in which self-sampling at home has been used to capture and follow changes in the circulating proteome.

References: [1] Deutsch et al (2021) J Prot Res: https://doi.org/10.1021/acs.jproteome.1c00657; [2] Suhre et al (2021) Nat Rev Genet: https://doi.org/10.1038/s41576-020-0268-2; [3] James et al (2020) Nat Med: https://doi.org/10.1038/s41591-020-01144-1; [4] Roxhed et al. (2021) Nat Comms: https://doi.org/10.1038/s41467-021-23893-4); [5] Fredolini et al (submitted)

AL65

Persistence of proteomes, targetable lesions, and therapy sensitivity through disease evolution in pediatric malignacies has implications for precision medicine

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Objective Timing is a serious challenge to precision medicine in pediatric cancer: how to identify and test personalized treatments for each child with relapsed or refractory disease in a timeframe that enables effective clinical intervention. We hypothesize that a change in personalized pediatric oncology from the current reactive to a proactive approach initiated at diagnosis is feasible if actionable genetic lesions and protein pathways are stable throughout disease progression.

Methods We use DIA based quantification of proteins, phosphorylation and proteolytic processing in patient biopsies and xenograft models integrated with next generation sequencing (NGS) and drug sensitivity measurements in retrospective cohorts paired with

published datasets. Acute Lymphoblastic Leukemia (ALL) progression samples from 11 patients & healthy donors were sourced from BC Children"s Hospital. DIA using a spectral library built by Gas Phase Fractionation and directDIA identifies >8000 Proteins on a QE HF. To asses stability in solid tumors of diverse pathology we re-analyzed published data and performed DIA proteomics on biopsies taken at multiple timepoints and metastatic sites. H&E stained FFPE sections were dissected into tumor and adjacent normal and processed using a new automated sonication-free FFPE workflow identifying >6000 proteins. Stability between patient and matched xenografted cells was assessed by integrated protein, phospho and HUNTER N termini analysis from 60µg of starting protein lysate. Persistence was measured using tests for statistical equivalence and difference. Drug sensitivity was measured in viably-frozen samples cultured and treated with drugs informed from published target-agent data.

Results 63% of proteins of interest in pediatric cancer showed significant equivalence between paired patient samples while only 7% differed significantly. The full proteome also showed highest equivalence of protein abundance between paired patient samples rather than similar disease states or random pairings. NGS indicated retention of variants in 10 of 11 BCCH patients or 56/80 patients including public data. Only 7/80 patients lack shared variants through disease progression. Murine xenografts accurately reflect the *in patient*proteome with notable exceptions in select pathways and more variability at the PTM level. Dx-R/R1-R2 samples from four ALL patients showed similar responses to four molecular-targeted drugs. Proteomics identified stable actionable candidates not identified at genome or transcript level and explained some drug sensitivities that diverged from genome-based prediction.

Conclusion Changes in variant frequencies mark disease evolution. But, paired ALL progression samples show stable proteomes, targetable lesions and therapy sensitivity. Thus, initiation of a precision medicine workflow when pediatric ALL is first diagnosed has the potential to identify disease sensitivities that are likely to persist at disease relapse.

AL69

Modular antibody de novo sequence analysis using multi-tier proteomics data

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[2]*Netherlands Proteomics Centre, Utrecht, Netherlands*

While mass spectrometry (MS) is the method of choice for sequencing protein mixtures de novo, sequencing polyclonal antibody (Ab) mixtures still poses a challenge[1]. Sequencing is typically performed by bottom-up (BU) methods, which are hampered by sequence homology of Ab sequences. Top-down (TD) MS, can be a solution by retaining the link between fragments and precursor, however the field is still maturing and de novo sequencing by TD MS remains challenging[2]. Still, BU and TD MS yield useful, complementary sequence information[1-3]. Here, we aim to combine BU and TD MS for automated de novo sequencing of Abs from polyclonal mixtures.

We select 5 template sequences per analyzed precursor chain using ETD LC-MS/MS of reduced Ab chains. These templates are used to untangle de novo peptide predictions (reads) for several multi-protease HCD/EThcD LC-MS/MS runs, grouping sequence information per Ab chain. Sequence tags in the TD spectra are extended using these reads to yield long confident sequence predictions (contigs). We assess these in a modular fashion based on germline domains. Finally, we recombine the results into full-length sequences.

We analyzed several samples of increasing complexity, namely monoclonal, mixtures of monoclonal, and polyclonal Ab samples.

For a monoclonal sample (TZB), filtering a V-region database of 250 with BU spectra yields 66 hits. We use TD MS fragments and a custom sliding window fragment matching algorithm to score the remaining V-regions. The top-5 best scoring V-regions are chosen as templates and have an average of 75% (heavy chain (HC)) and 85% (light chain (LC)) sequence identity. A C-region is selected based on direct mass matching of C-terminal fragments, and a J-region is selected through alignment to BU reads. An accurate reconstruction of the target sequence was achieved for both the LC and HC (excluding 5 I/L errors) by reconstructing the framework region sequences and filling the remaining gaps with mass matching BU reads.

Similar results were obtained when TZB was analyzed in a mixture with two monoclonal Abs. When processing a clone from serum from a sepsis patient, similarly accurate templates were obtained. While long, accurate (>95%) contigs were obtained, contaminant clones hampered getting complete Ab sequences in a fully automated manner. We were however able to obtain the sequence through manual inspection of the results.
This pipeline aims at automated sequencing of Abs from polyclonal mixtures with multi-tier MS. It enables the data-driven assignment of reads to precursor chains, reducing complexity of sequencing.

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Captions
1: Overview of the experimental workflow
2: Overview of the analytical pipeline

Fig. 1

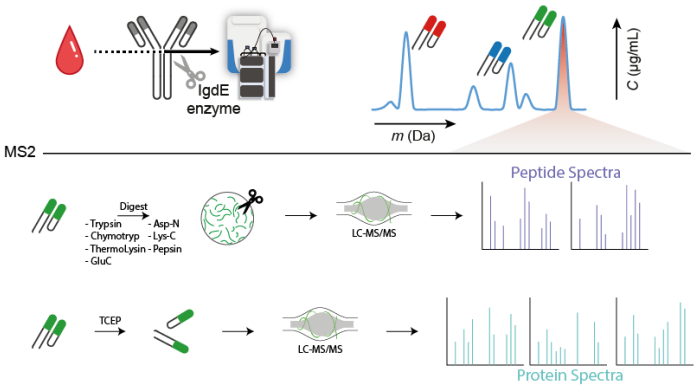
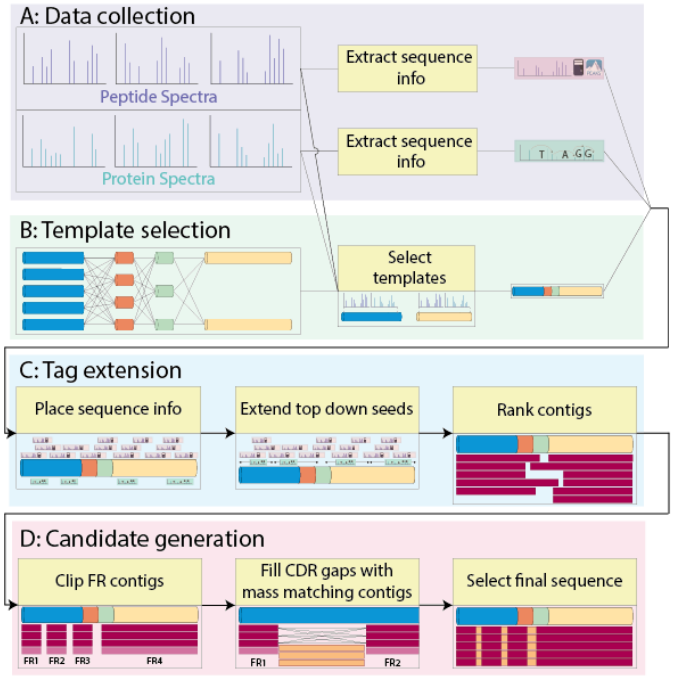


Fig. 2



AL70

High sensitivity immunopeptidomics using an optimized DDA-PASEF allows the identification of MHC-ligands of the SARS-CoV-2 Spike protein

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Objective: Immunopeptidomics characterizes the identity and dynamics of peptides presented on major histocompatibility complex (MHC) molecules and recognized by T lymphocytes to combat infections and tumors. Identifying disease- and tumor-related immunopeptides is critical to develop vaccines and targeted immunotherapies. Here, we aimed to optimize a high coverage immunopeptidomics workflow, harnessing the additional separation dimension of trapped ion mobility spectrometry (TIMS) in the TimsTOF-Pro-2 platform.

Methods: Cell pellets of JY human cell line wild-type or expressing the SARS-Cov-2 spike protein were lysed by sonication in 1% CHAPS in PBS buffer and MHC-peptide complexes were immunoaffinity-enriched using W6/32 anti-human-MHC-A, B, C antibody coupled to CNBr-activated agarose beads. After washing with PBS and

eluting with 0.2% trifluoro-acetic acid, peptides were ultrafiltered (10 kDa cutoff) and desalted. NanoLC-MS analysis was performed using a nanoElute coupled to timsTOF-Pro-2 injecting the equivalent sample to 7 Million cells in a 2-hours RP chromatographic separation in DDA-PASEF with different parameters to optimize the MS acquisition. Database search was performed in PEAKS XPro using unspecific cleavage, with a cutoff of 1% FDR. Data analysis was performed in R and predicted MHC-binding affinity was evaluated using NetMHC 4.0 and GibbsCluster 2.0.

Results: We developed an optimized DDA-PASEF method for the identification of lowly-abundant MHC class I ligands. The optimized workflow effectively doubled the number of peptides identified compared to the standard method. Notably, the raw area CV across 3 replicates was below 20% for 70% of the peptides identified across 3 technical replicates, showing a high quantitative reproducibility. Furthermore, adapting the MS/MS polygon filter for MS/MS acquisition, the confident identification of +1 peptides was possible, which accounted for 20% of the peptides identified. The fully optimized method allowed the identification of approx. 10,000 peptides from an equivalent of less than 7 million cells. Of the identified peptides, more than 85% were predicted to bind human MHC class I complexes with low- or high-affinity. In conclusion, our optimized workflow achieved a performance similar to state-of-the-art methods, which typically require analyzing at least 15-fold the starting material used in our study. The achieved level of sensitivity allowed us to detect putative MHC-ligands specific to the SARS-CoV-2 Spike protein and sets the basis for characterizing the immunopeptidome of scarce samples such as tumors and cancer biopsies and constitutes and essential step towards the development of personalized immunotherapies.

Conclusions: We developed a highly sensitive and reproducible DDA-PASEF immunopeptidomics method for the identification of MHC class I ligands which will enable the study of neoantigens of cancer-related mutations from scarce-samples such as tumors and cancer biopsies.

AL71

A bead-based immunoproteomic assay (Infection Array) to monitor pathogen-specific antibody responses in periprosthetic joint infection (PJI)

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Objectives: Periprosthetic joint infections (PJIs) remain the most serious complication of modern arthroplasty. Diagnosis of PJI and pathogen identification pose considerable challenges in clinical practice, which can delay optimal antimicrobial therapy. We hypothesize that the pathogen-specific immune response to PJI reflects the infection process, provides clinically relevant information and has the potential to further optimize antimicrobial therapy. To test this, we conducted a prospective matched-cohort pilot study in PJI patients undergoing two-stage septic revision surgery and in non-infected controls. **Methods:** We developed an Infection Array (IA), a custom Luminex®-based quantitative high-throughput state-of-the-art bead-based suspension array for simultaneous measurement of multiple antibody specificities. The IA was prepared by cultivating 30 pathogens commonly associated with PJI and purifying their extracellular proteins. These complex extracellular protein extracts were then coupled to magnetic microspheres (MagPlex beads) with unique spectral signatures. Nine PJI patients undergoing two-stage septic revision surgery between 06/2020 and 06/2021 were matched to ten control patients undergoing aseptic revision surgery. Pre-, intra- and postoperative samples were collected at multiple standardized time points. To study both long-term and acute antibody production, we collected serum samples, as well as EDTA-blood that was used to isolate peripheral blood mononuclear cells (PBMCs). The washed PBMCs were seeded into cell culture plates for the collection of medium enriched for newly-synthesized antibodies (MENSA), i.e., cell culture supernatant containing freshly produced antibodies. Over the course of this pilot study the pathogen-specific antibody responses were measured using the IA in 699 patient samples (237 serum and 462 MENSA samples), and the data were analyzed using the xMAPr app. **Results:** The IA was able to trace the dynamics of the pathogen-specific humoral immune response to PJI

in serum and MENSA. The pathogen-specific serum antibody titers declined in PJI patients over the course of the treatment, while no changes in antibody titers were observed in the aseptic control patients during this study (40 – 395 days). Moreover, in all patients, acute antibody responses were observed against a number of PJI-related pathogens, prominently *Staphylococcus (S.) epidermidis*, *S. warneri* and *Candida albicans*. **Conclusions:** The IA is a promising tool of high resolution for monitoring the immunoproteomic footprints of infectious agents during the course of PJI. Our results provide insights into the pathophysiology of PJI, as well as information about the individual disease course that could be of clinical value. In the future, immunoproteomic analysis of the patients" long-term and acute antibody responses may complement the standard diagnostic portfolio of PJI and ultimately help to monitor and improve the outcome of PJI treatment.

Poster presentation

P01

Ultra-sensitive proteome quantification on the timsTOF SCP mass spectrometer

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Introduction

Single cell proteomics is a relatively young niche of proteomics compared to single cell genomics. In the recent years, significant progress has been made in sample handling and boosting the sample signal by multiplexing with mass spectrometers. timsTOF SCP is the first commercially introduced mass spectrometer dedicated for single cell proteomics. The modified front-end (orthogonal ion-guiding) of the instrument increase the ion transfer up to five times and keeps ultra-high robustness – the default attribute of the timsTOF platform. Here we demonstrate the performance of the instrument for low sample loads in the range of 250 pg to 1 ng in combination with robust low flow rate delivery from the Evosep system.

Methods

Commercially available K562 tryptic peptide digests (Promega) were loaded on the Evotips according to the recommended protocol provided by the vendor. Peptide amounts from 125 pg to 25.6 ng were used to evaluate the performance of the instrument. The Whisper low flow method – 40 samples per day (SPD) with a gradient time of about 28 minutes was used for separating the eluting peptides on a 15 cm column with 75 µm ID coupled to captive spray ionization source using a 10-micron ID zero-dead volume emitter. Samples were analyzed using a dia-PASEF method tuned for high sensitivity measurements. Raw data were processed in Spectronaut 15.

Preliminary Data

Processing of diaPASEF data was performed with Spectronaut 15 using a spectral library of 5,200 protein groups and 54,000 peptides and a further improved library with 9,300 protein groups and 116,000 peptides, respectively. Using the combination of Whisper 40 SPD and the high sensitivity methods on the timsTOF SCP, we could quantify around 1,500 protein groups from 250 pg sample loads and close to 2,000 protein groups from 500 pg loads. Performing a dilution series experiments, around 4,500 protein groups could be quantified in 6.4 ng peptide loads.

Fig. 1

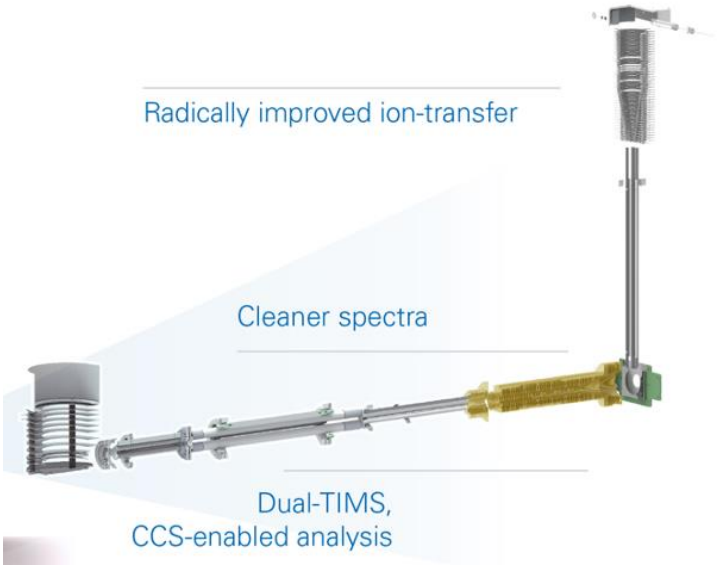
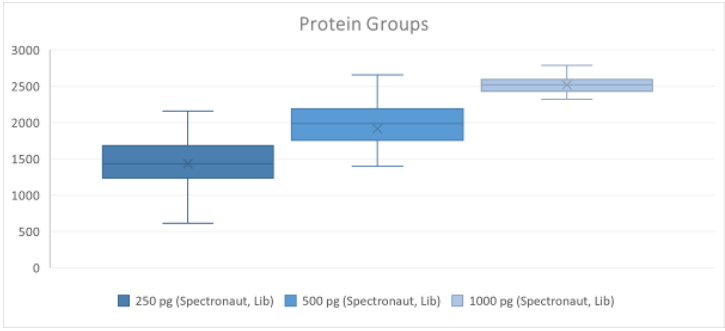


Fig. 2



P03

Sensitivity gains in nano-flow and ultra-low nano-flow LC-MS: practical considerations for limited sample proteomics

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Maximizing the LC-MS sensitivity is essential for bottom-up proteomics application, especially in single-cell proteome analysis where sample amount is limited. Several technologies have been developed either using split flow or conventional 75 µm I.D. column with lower flow rates, which, however, either induced sample loss or sub-optimal sensitivity, respectively. Here we report an automatic splitless

ultra-low nano-flow LC-MS approach at 60 nL/min with a 25 µm I.D. × 15 cm column to reach optimal sensitivity and throughput for single-cell proteomics application.

We thoroughly studied the effect of flow and column ID and length on MS sensitivity, peak capacity, and the number of protein and peptide identifications. The critical for ultra-low nano-flow LC-MS components of LC and MS were optimized to achieve maximum separation quality and proteome profiling results. The new low-flow LC system allowed to allow low volume sample injections (from 10 to 100 nL) used to enable precise quantification for limited sample amounts. The sensitivity improvements up to 10-folds for HeLa cells protein digest in comparison with standard nanoLC-MS experiments was achieved by utilizing flow rates below 100 nL/min at UHPLC conditions and columns with internal diameter 20-30 µm. The further optimization enabled ca. 30 times sensitivity gains and throughput comparable with fast nanoLCMS analysis. The robust system operation and low-flow rates and high-pressures provide new possibilities for automated high-throughput single-cell proteomic analysis.

Compared to the 75 µm I.D. column at an optimal flow rate of 200 µL/min, this novel setup reaches up to 32 times higher sensitivity and therefore, enables the highest number of protein and peptide identifications. Overall, this novel method significantly improves the nanoLC-MS sensitivity, enables 12 and 24 samples per day measurement, and is highly feasible for single-cell proteomics analysis while combined with tandem tags labeling.

Fig. 1

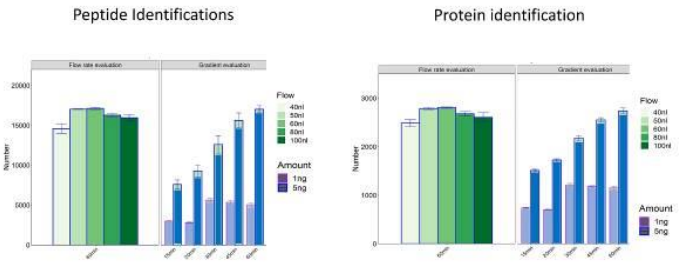

Figure 1. The effect of flow rate on the number of peptide and proteins identified.

P04

Number of detected proteins as the function of the sensitivity of proteomic technology in human liver cells

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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. 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 re-action monitoring and shotgun methods of MS analysis using one-dimensional and two-dimensional fractionation to identify the proteins encoded by human chromosome 18. A total of 134 proteins were identified. The overlap between proteomic and transcriptomic data in human liver tissue was ~50%. This weak convergence is due to the low sensitivity of proteomic technology compared to transcriptomic approaches. Data is available via ProteomeXchange with identifier PXD026997.

P05

Global targeted protein complex detection by Mini-Complexome Profiling (mCP)

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Protein complexes are key components of cellular and physiological processes. The disruption or alteration of these complexes due to structural, functional or localization changes of even a single protein has been correlated to cardiac diseases as for example in Atrial Fibrillation (AF)1. Many efforts have been made to study protein complexes in a global manner2–4. Two main approaches are used for protein complex detection5, the detection of protein complexes by unbiased hierarchical clustering and targeted approach. The study of protein complexes by targeted approaches has been recently developed3. The targeted approach is based on previous protein complexes database information. It allows the detection of hundreds of protein complexes in a cell or tissue by a system biology approach, but it is time consuming and expensive when performing exploratory comparison experiments.

Here we present a reduced workflow, mCP, for targeted detection and comparison of proteins complexes between two or more different cell type conditions. This workflow allows an exploratory and systematic comparison of protein complexes while requiring less number of fractions per condition in comparison to the already developed targeted approach3. The workflow includes: mild extraction of protein complexes, electrophoretic separation, and fractionation by mini-Blue Native PAGE (BN PAGE), Data Independent Acquisition (DIA) mass spectrometry; and bioinformatics targeted detection by a custom statistical software script, written in R (mCP-R).

We applied our protocol to study changes in the complexome due to the absence of the phospholamban (PLN) gen. Genetic alterations in this gen, like deletion of Arg14 in human PLN are strongly associated with a cardiac disease called Dilative Cardiomyopathy (DCM)6. We were able to detect more than 30 protein complexes in mouse ventricular cardiomyocytes knockout (KO PLN) and wild-type conditions. Protein complexes were validated by manual, script-assisted validation as well as correlation between the two biological conditions. The resulting data point to a possible functional connection between PLN and Caveolin-3 (CAV3) in mouse ventricular cardiomyocytes, however pending validation by independent experimental approaches.

Note: This research is supported by DFG Collaborative Research Centre 1002, Modulatory Units in Heart Failure, University Medical Center Goettingen and Georg-August-University of Goettingen, Germany.

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P06

Targeted Quantification of the Human and Mouse Lysosomal Proteome

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Objective

Lysosomes play a major role in cellular metabolism, catalyzing the degradation/recycling of extra- and

intracellular material, and acting as metabolic sensory system as well as signaling hub. This is facilitated by the lysosomal proteome, consisting of ~340 luminal, membrane, and associated proteins. The reproducible quantification of these proteins is a prerequisite to study the lysosome`s involvement in disorders and cellular processes. Due to its low abundance, organelle enrichment is necessary for an unbiased analysis, restricting the number of potential samples. To enable the quantification of the lysosomal proteome from various sources, we established targeted assays for its relative and absolute quantification.

Methods

Samples were digested in solution using RapiGest. Standards were generated following the quantification concatemer (QconCAT) strategy, expressed in SILAC media using *E. coli*, purified, digested, and quantified. The following digestion methods were tested: FASP, SP3, in gel, and in-solution using GCl or RapiGest. Samples were analyzed by MRM, PRM, and DIA using an Orbitrap Fusion Lumos and a QTRAP 6500+. Data were analyzed with Mascot/Proteome Discoverer, Spectronaut, Skyline, Panorama AutoQC, and mProphet.

Results

We established scheduled PRM assays for the human and mouse lysosomal proteome, manually selecting suitable peptides based on their properties and signal intensities in re-evaluation experiments from >18,000 mouse/human candidate peptides. This resulted in 586/824 peptides covering 340/301 lysosomal proteins for mouse/human samples, respectively. Following retention time re-assignment using iRTs, scheduled assays were designed on an Orbitrap Fusion Lumos consisting of one/two runs of 120/60 minute gradients, respectively, and applied to lysosome-enriched fractions and whole cell/tissue lysates. Using mouse samples, we compared the performance of DIA and PRM, for which the latter showed a better performance, especially for whole tissue lysates. To further allow for the absolute quantification of the mouse lysosomal core proteome, we designed, optimized, expressed, and purified 12 QconCAT proteins, containing 399 surrogate peptides for 142 lysosomal proteins. Following digestion optimization and peptide re-quantification, we optimized spike-in amounts and generated scheduled assays on a QTRAP6500+. We absolutely quantified lysosomal proteins in FACS-counted lysosomes, 5 cell lines, and 9 mouse tissues from wild type samples, and tissues of mouse models for Alzheimer"s Disease, Pompe Disease, and mucopolysaccharidosis III A-C. In both cell and tissue samples, we observed a ~2000-fold dynamic range in protein copy numbers within one cell/tissue type, and a ~3 fold dynamic range with respect to total lysosomal proteins amount between tissues.

Conclusion

We established targeted assays for the relative and absolute quantification of the lysosomal proteome in human and mouse samples.

P07

CLINSPECT-M – Results from the first Best Practice Proteomics Ring Trial for clinical specimen

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The foundation for integrating mass spectrometry (MS) into systems medicine is to develop standardized start-to-finish and fit-for-purpose workflows for clinical specimen. An essential step in this pursuit is to capture best practices for MS-based proteomic measurements in order to highlight common ground in a diverse landscape of various sample preparation techniques and LC-MS set-ups, to explore differences and potential confounding factors as well as to identify areas for improvement with a direct practical benefit.

At six different study centers participating in the CLINSPECT-M consortium (https://clinspect-m.mscoresys.de) pooled plasma, serum, cerebrospinal fluid (CSF) samples and glioblastoma FFPE tissue were analyzed with the respective current best preparation protocols and LC-MS settings and set-ups. For each sample type 10 replicate DDA measurements per LC-MS set-up were performed and in some cases DIA measurements were also conducted. The resulting data was collected and analyzed with a standardized pipeline. The various preparation and LC-MS set-up combinations were compared in several aspects such as the number of identifications (IDs), data completeness, overlapping IDs as well as quantitative and retention time precision.

For the analysis of plasma and serum the results show a high degree of similarity between centers despite distinctions in preparation and LC-MS set-ups. Especially on proteingroup level all results revolve around 260 IDs for both serum and plasma. In the case of the CSF and FFPE, differences between the number of IDs are more evident and are reflected in a discrepancy up to around 800 and 2000 proteingroups from lowest to highest value for CSF and FFPE, respectively. From a broader perspective, all LC-MS set-ups demonstrated good quantitative and retention time precision independent of the measured sample type. Additionally in many cases, DIA measurements display a better data completeness in contrast to DDA measurements, whereas the difference diminishes from precursor to proteingroup level.

This multicenter round robin study provides an attractive snapshot of what is currently available in the CLINSPECT-M consortium for measuring these types of clinical specimen and hence the results of each individual preparation LC-MS set-up combination serves as valuable reference point for laboratories with similar protocols and settings.

P08

Proteome profiling and proteogenomics reveals molecular re-arrangement and differential proteolysis in a mice model of reverse cardiac remodeling

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Objectives: Currently, left ventricular assist device (LVAD) implantation is one of the two available therapies after heart failure (HF). However, in a small subset of patients, LVADs promotes cardiac unloading and recovery of heart function, allowing subsequent LVAD explantation. Characterizing the molecular mechanisms associated with reverse cardiac remodeling (RCR) is crucial for the identification of predictive markers of cardiac recovery. Through mass spectrometry (MS)-based proteomics analyses on a mouse model, we explored the quantitative dynamic of both proteogenomic signatures and post-translational modifications associated with RCR (Fig. 1).
Methods: Left-ventricular (LV) samples were obtained from 20 mice subjected to transaortic constriction (TAC-HF); in a group of mice, the constriction was removed (rTAC), resulting in nearly complete cardiac recovery. Proteins were trypsin-digested, TMT-labelled, pre-fractionated, and analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Spectral data were processed via the FragPipe bioinformatic pipeline. For general proteomics and proteolytic analyses, a semi-tryptic search was performed against a mouse reference proteome. An in-house-developed approach was used for the analysis of proteolytic products. Two proteogenomic approaches were explored. A protein sequence database of predicted canonical and variant open reading frames (ORFs) was generated from ribosome footprinting experiments using RiboTag analysis of cardiomyocytes from a TAC model; and a custom database was generated from publicly available RNA-seq data from mouse TAC models. Principal Component Analysis (PCA) and linear models were applied to identify differentially expressed proteins and enrichment analyses were performed to characterize the biological motifs of differentially abundant proteins.
Results: The general proteomics analysis identified and quantified 3959 proteins. PCA shows a defined separation between experimental groups. Of note, the

Poster presentation

LV proteome is more similar to the control group than the TAC group. After a semi-specific search, >5K proteolytic products were identified. PCA on these features segregates the TAC group, and 162 of these proteolytic products are differentially abundant (Fig 2). Proteogenomics analyses identify 132 peptides uniquely predicted by RiboTag and 30 single-amino acid variants from a phenotype-specific database. PCA on these non-canonical features allows to segregate TAC vs SHAM (control) groups, suggesting their potential functional role during HF. **Conclusion:** The use of MS-based proteomics in combination with sequencing information and the extensive exploration of the spectral data, reveals the molecular re-arrangement of cardiac tissue after HF and RCR in a mouse model, offering novel mechanistic insights into this process and the potential for the discovery of biomarkers of cardiac recovery in LVAD patients.

Fig. 1

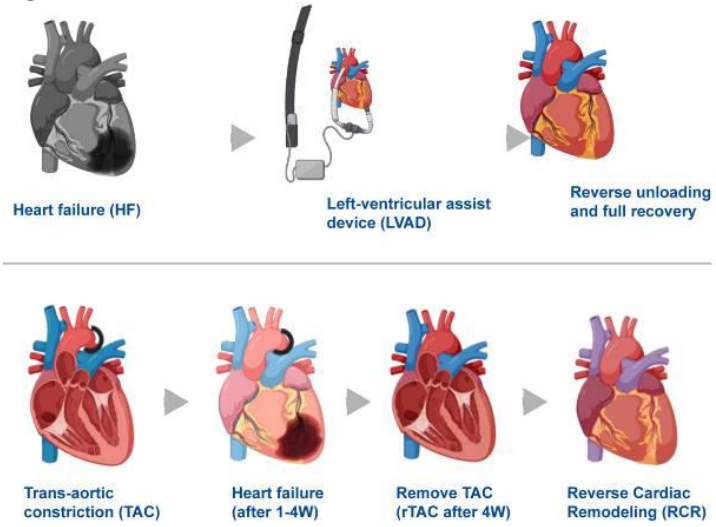
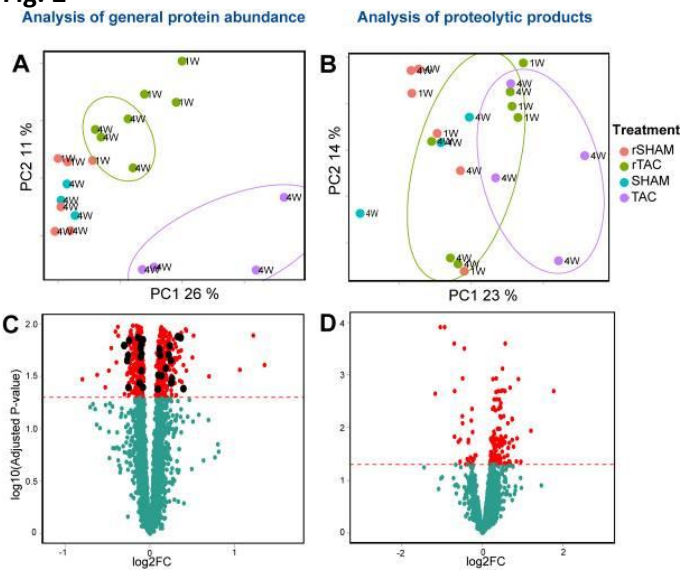


Fig. 2



P09

Subcellular proteomics of antibody-producing CHO and plasma-cell derived cell lines

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Today, recombinant biotherapeutics play an essential role in the treatment of severe illnesses like various cancer types or autoimmune diseases. The first choice cell lines employed to meet the constantly growing demand of these pharmaceuticals are Chinese hamster ovary (CHO) cells. Thus, the cell lines are under constant development in order to increase recombinant product yield. With the advent of large-scale omics technologies, the last decades saw a shift from empirical, media-driven CHO cell line optimization towards rational cell engineering. Especially advances in high-resolution mass spectrometry enabled deep proteome coverage and high data quality. However, the spatial aspect of global proteome regulation in CHO and antibody-producing cells in general mainly remained unadressed. Here, we present the first global subcellular proteome of an antibody-producing CHO-K1 cell line and compare the spatial protein distribution to a murine plasma-cell derived (PCD) cell line in order to detect differentially regulated pathways associated with antibody secretion. Both cell lines showed endoplasmic reticulum structures highly adapted to protein secretion, as immunoglobulin chains were found co-localized to chaperones and proteins involved in protein glycosylation. Differences in the subcellular distribution of mammalian target of rapamycin complex 1 (mTORC1) associated proteins as well as Rab proteins involved in secretory vesicle trafficking suggest that the cell lines rely on different pathways in order to accomplish high protein secretion. This study highlights the potential of subcellular proteomics as workflow to identify characteristics of high recombinant protein production in production cell lines.

P10

The qualitative and quantitative composition of the surfaceome during chondrogenesis

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Objective: The surfaceome is the assembly of plasma membrane and cell surface molecules that regulate their function and phenotype, and maintain communication with the extracellular matrix (ECM). Plasma membrane proteins perform key functions including cell-matrix interactions, communication, receptor signalling, and transport of ions and other molecules. Despite the emerging role of the surfaceome in mature chondrocyte metabolism, proliferation and differentiation, many open questions remain concerning their involvement in cartilage formation. Chondrogenesis is the process whereby progenitor cells differentiate into chondroblasts and then chondrocytes. Given that the ECM of developing cartilage undergoes profound changes, we hypothesised that differentiating progenitor cells are characterised by a dynamically changing surfaceome. There is currently a lack of knowledge about the chondroprogenitor surfaceome during chondrogenesis. The purpose of this study was to determine the qualitative and quantitative composition of the surfaceome during chondrogenic differentiation, and to identify biomarkers characteristic to each major step of the process.

Methods: Chondrifying cell cultures were established from distal parts of limb buds of early chicken embryos. On specific days of chondrogenesis, membrane proteins were labelled, enriched and isolated using an aminooxy-biotinylation (AOB) technique and analysed by mass spectrometry using high throughput shotgun proteomics. Cell surface proteins were classified into functional groups based on Gene Ontology (GO) molecular functions.

Results: Using the AOB labelling approach, a total of 241 unique proteins could be identified ($P<0.05$), of which 154 proteins were classified as surface proteins (64%). 104 proteins (68%) were detected in day 1 cells, 114 (74%) were identified in day 3 cells, 152 (99%) were detected in day 6 cells, 148 (96%) were identified in day 10 cells, and 143 (93%) were detected in day 15 cells. 95 (62%) were common between all culturing days. 55 proteins were classified as enzymes (36%), 45 proteins had receptor roles (29%), 15 proteins were involved in transport processes across the PM (10%), 16 proteins were involved in adhesion, cell-cell or cell-matrix junctions and cytoskeletal organisation (10%). 52 proteins (34%) could not be assigned to the subgroups, or their function was unknown.

Conclusion: Our results provide proteomic data on differentially expressed low-abundance membrane proteins on the surface of differentiating chondrocytes. Identifying biomarkers specific to the main stages of cartilage formation may offer novel means by which osteo-chondroprogenitor stem cells could be directed towards the chondrogenic lineage, thus generating better cartilage for tissue engineering.

Financial support: Young Researcher Excellence Programme (FK-134304) of the National Research, Development and Innovation Office, Hungary; TKP2020-NKA-04; EFOP-3.6.3-VEKOP-16-2017-00009.

P11

Proteomic analysis of the surfaceome in human cutaneous melanocytes and melanoma cells

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Objective: Cell surface proteins, which collectively constitute the "surfaceome" fraction of the proteome, play crucial roles in determining cellular phenotype and identity. The qualitative and quantitative composition of the surfaceome undergoes alterations during normal and pathological differentiation. There are currently no data available concerning the complement of cell surface proteins of normal and pathological human pigment cells. Therefore, this project is aimed at discovering the melanocyte and melanoma-specific cell surface proteome.

Methods: Three pigment cell cultures were used in our experiments: primary epidermal melanocytes (from human skin), WM35 cell line (from *in situ* melanoma), and A2058 cell line (from metastatic melanoma). Cell surface proteins were selectively labelled by aminooxy-

biotin and then isolated by streptavidin-conjugated beads. Isolated proteins were trypsin-digested and peptides were analysed by high-throughput shotgun mass spectrometry. Identified proteins were then analysed using bioinformatics tools and manually curated. For validation, we performed RT-qPCRs, western blots and immunocytochemistry reactions.

Results: Based on GO annotations 70-84% of the identified proteins were found to be cell surface proteins. A total of 416 (melanocyte), 256 (WM35) and 567 (A2058) surface proteins were positively identified during the analysis. The combined list of proteins comprised 629 different proteins, 128 of which were detected in melanocytes only, whereas 338 proteins were specific to the melanoma cells and 164 proteins were identified in all three cell types. Further analysis revealed the ratio of functionally relevant proteins (enzymes, receptors, transporters, adhesion proteins) within the surfaceome. Based on the proteomic analysis we found several proteins worth of further examination, but our RT-qPCR analysis is still in progress. However, we singled out two proteins during our validation process: proteomic results identified basigin (CD147) in melanoma cell lines only, while gasdermin D (GSDMD) was exclusively present in melanocytes. Western blots on plasma membrane fractions strengthened our proteomic findings in terms of basigin, but GSDMD was detected in all three cell cultures. Surprisingly, immunocytochemistry reactions followed by confocal microscopic evaluation revealed basigin and GSDMD expression in all three cell cultures, although signals seemed to show opposite intensity levels in healthy vs malignant relations.

Conclusions: The differences found in the surfaceome of healthy vs malignant pigment cells can lead to the discovery of highly selective biomarkers of disease onset and progression. The protein expression analysis verified our proteomic findings as basigin and GSDMD may become potential biomarkers in melanoma treatment.

Supported by grants: FK-134304; ÚNKP-18-2, ÚNKP-19-2, ÚNKP-20-2; EFOP-3.6.3-VEKOP-16-2017-00009

P12

Temporal Analysis of Protein Ubiquitylation and Phosphorylation During Parkin-dependent Mitophagy

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Parkinson"s disease is a severe neurodegenerative disease in which the selective degradation of mitochondria (mitophagy) is impaired. The complex interplay of the cytosolic E3 ligase parkin and the mitochondrial outer membrane (MOM) kinase Pink1 on MOM proteins is vital for the correct execution of mitophagy. Investigation of well-studied chronologically early events of mitophagy, e.g. post-translational modification of MOM proteins as well as so far under investigated late events, such as degradation of damaged mitochondria, are therefore needed.

We used quantitative proteomics to study the interplay of the proteome, ubiquitylome and phosphoproteome during early (2-6h) and late (12-18h) stages of mitophagy in Parkin wild-type (WT) or ligase-dead expressing HeLa cells. To this end we combined dimethyl labeling with phosphopeptide enrichment and GlyGly remnant enrichment with on-antibody tandem-mass tag (TMT) labeling. Samples were analyzed on Q Exactive HF or HF-X hybrid quadrupole-orbitrap mass spectrometer and downstream data processing and statistical analysis were performed by using the MaxQuant and Perseus software suits.

With our quantitative proteomics approach, we found an outside-in directed degradation process of mitochondrial subcompartments during parkin-dependent mitophagy. During early stages of mitophagy (2-6h), preferentially MOM and intermembrane space proteins are degraded. Only during later stages (12-18h), degradation of inner mitochondria membrane (MIM) and matrix proteins was detected. Interestingly, a similar outside-in progression of parkin-dependent ubiquitylation could be revealed. Within this process, several mitochondrial proteins of the outer (e.g. MFN1/2 or TOM70) and inner membrane (e.g. PHB2, TIM13) are initially labeled by parkin-dependent ubiquitylation before protein degradation is executed. In general, only a low

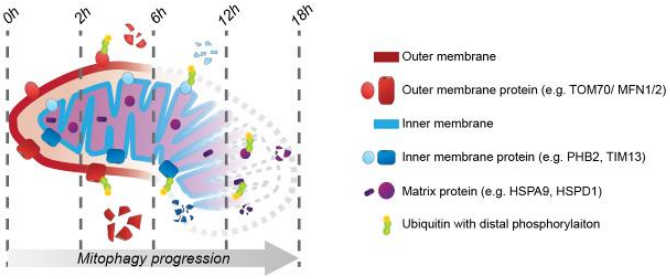
number of mitochondrial proteins revealed simultaneous ubiquitylation and phosphorylation during the progression of mitophagy. However, on the MOM protein VDAC2 we identified a cross-talk between phosphorylation and ubiquitylation that ultimately leads to protein degradation during parkin-dependent mitophagy.

In this study we used Parkin WT or ligase dead expressing HeLa cells, which allowed us to investigate besides early stages also late stages of mitophagy. We found a step-wise degradation of mitochondrial proteins, initiated at the MOM and proceeding to the inner subcompartments. These outside-in directed progression was also extended to parkin-dependent protein ubiquitylation. Our findings support the so far under investigated alternative mitophagy progression theory, in which mitochondria are not engulfed and degraded as a whole, but are stepwise marked and degraded in a defined order.

figure legend:

Outside-in ubiquitylation of mitochondrial proteins precedes protein degradation during parkin-dependent mitophagy

Fig. 1



P13

Defining the human peroxisomal proteome using spatial proteomics

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Objectives

Peroxisomes are metabolic organelles that perform a variety of essential cellular functions, including the β-oxidation of fatty acids, detoxification of hydrogen peroxide, biosynthesis of plasmalogens and bile acid, and signaling. They share metabolic pathways and form membrane contact sites with other organelles. Deficiencies in peroxisome biogenesis or function have been linked to severe, often lethal human diseases such as the Zellweger spectrum disorders. To fully understand peroxisome functions, dynamics and crosstalk with other organelles, knowledge of the complete peroxisomal proteome is mandatory. However, due to the small size of peroxisomes, their low abundance, shared localization of proteins, and the formation of physical tethers to other organelles, defining the peroxisomal proteome is challenging. So far, about 140 proteins have been linked to human peroxisomes, many of these being dual- or multi-localized. In this study, we aim at defining the human peroxisomal proteome including multi-localized and membrane contact site proteins following a spatial proteomics approach combined with machine learning for data analysis.

Methods

We established a workflow for the isolation of peroxisomes from human cells (HEK293) employing subcellular fractionation and density gradient centrifugation. Using high-resolution LC-MS, we profiled the distribution of cellular organelles across subcellular and density gradient fractions. Based on profiles of established marker proteins for different subcellular niches, we trained support-vector machine learning models to identify new peroxisomal proteins. For the identification of dual-localized peroxisomal proteins, we performed protein-correlation profiling using selected dual-localized marker proteins.

Results

We profiled more than 10,000 proteins and identified most of the known peroxisomal proteins as well as novel candidates. Our data confirm that the majority of peroxisomal proteins is dual- or multi-localized. Furthermore, our data demonstrate the high potential of our approach to identify peroxisome-associated contact site proteins, exemplified by the assignment of the known ER-peroxisome contact site protein VAPB (Costello et al., 2017), residing in the ER membrane, to both peroxisomes and the ER. Based on the correlation between the profiles of marker and candidate proteins, we chose promising novel peroxisomal candidate proteins that are currently being validated using independent methods.

Conclusion

Taken together, the results of our subcellular profiling approach represent a milestone towards defining the human peroxisomal proteome including multi-localized proteins. Our dataset will serve as an important resource for future studies of peroxisome biology and functions and for elucidating the role of peroxisomes in human health and diseases.

P14

Peptide Mass Spectrometry Imaging (MSI) of Pancreatic Cancers and the Application of Open Source Analysis Tools for the Establishment of a Powerful and User-friendly Analysis Pipeline

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Introduction

Mass Spectrometry Imaging (MSI) allows analyzing tissue specimens for the spatial distribution of hundreds of biomolecules such as proteins, peptides and metabolites. It"s a powerful tool for clinical pathology as it can assist in generating diagnoses and prognoses via the measurement of molecular and morphological tissue properties. The frequent pancreatic ductal adenocarcinomas (PDAC) show limited therapy options and patients suffer from late diagnosis due to vague symptoms. The rather rare endocrine pancreatic cancers (PNET) show a slightly better prognosis but are divided into a large number of subtypes. We applied MSI of peptides to identify m/z features distinguishing between PDAC and PNET. Using open source analysis tools that were previously implemented in the Galaxy environment, we established a powerful analysis pipeline that provides an easy to handle user interface and complies to guidelines for reproducible and transparent analyses.

Methods

We performed MSI on a cohort of PDAC and diverse PNET cases aiming at identifying spatial peptide clusters, which define the specific tumor areas and distinguish between PDAC and PNET. Two biopsies from 104 patients were assembled in 13 TMAs and measured on a rapifleX device (Bruker Daltonics). Data analysis was performed using the Cardinal and MALDIquant packages implemented in the Galaxy environment. To perform supervised classification analysis, tumor regions were annotated using GIMP and filtered with an established co-registration pipeline. M/z features were clustered using the spatial shrunken centroids algorithm.

Results

First analysis, focussed on the data preprocessing and quality assessment revealed a good data quality and identified ~ 750 m/z features that were used for supervised classifications. The classifier distinguishing PDAC and PNET showed an accuracy above 70 % and consisted of ~ 50 m/z features to determine PDAC and ~ 100 m/z features to determine the more diverse group of PNET. By the aid of LC-MS/MS data, we want to identify the m/z features of interest and report the corresponding peptides.

Conclusion

To conclude, we provide a powerful analysis workflow for transparent and reproducible MSI data analysis in Galaxy and show its applicability to a large clinical dataset. Furthermore, first results highlight the power of MSI to study peptide profiles and establish a spatial classifier to identify PDAC tumors in a set of different pancreatic cancers.

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P15

Twenty Thousand Leagues in the North Sea: a proteomics perspective into recurring bacteria during algal blooms

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Microalgae flourish in marine ecosystems along coastlines due to seasonal changes and agricultural run-offs. Algal species contribute to at least half of the global photosynthetic carbon fixation. During and following the algal bloom, the nutrients released in the seawater provide favourable conditions for bacterioplankton to grow. Using carbohydrate-active enzymes (CAZymes) specific to algal polysaccharides, these bacteria have the metabolic potential to thrive in the environment and remineralise carbon fixed in the algal bloom. We investigate this nutrient cycle at the long-term sampling site "Kabeltonne Helgoland" in the North Sea. First insights into the samples revealed the dynamic composition of bacterioplankton in response to algal bloom, which largely consists of a diverse array of Flavobacteria. Therefore, the present study focuses on an environmental bacterium, Polaribacter sp. KT25b, within the Flavobacteria, and its key proteins involved in algal polysaccharide degradation. Here, we test a proteomic sample preparation workflow for low cell numbers to mirror the conditions found in marine ecosystems. For an in vitro analysis of proteins from 1 million Polaribacter sp. KT25b cells, we employed SP3-single pot, solid-phase sample preparation (SP3) technique and compared it to in Stage tip (iST) kit method. Liquid chromatography with tandem mass spectrometry analysis integrated with data processing using experimental spectral library reveals a better identification with the SP3 technique than the iST kit method. SP3 technique improves identification with each biological replicate, yielding more than 200 proteins as opposed to 150 proteins obtained with the iST kit method. In the future, this workflow can be combined with cell-sorting experiments targeting Polaribacter spp. cells from environmental seawater samples. Finally, this will aid in gaining a deeper understanding of the metabolic potential of marine bacteria at finer-grained taxonomic and temporal resolution.

P16

Absolute quantification of influenza A virus proteins during single-round replication of MDCK suspension cells

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Objectives: Madine-Darby canine kidney cells are widely used as a host for influenza vaccine production. To improve production yields, a detailed understanding of the virus life cycle and virus replication is crucial. For mathematic modeling approaches, quantitative data is required for the characterization of processes and dynamics. So far, most single-cell models are mainly based on information about viral RNAs dynamics. Quantitative data regarding intracellular protein expression is more or less missing but could add valuable information regarding the timing of virus-induced changes during the viral life cycle. Therefore, the aim was to develop a mass spectrometry-based assay for the absolute quantification of four major influenza A virus proteins (matrix protein 1, nucleoprotein, neuraminidase, hemagglutinin) for single-round replication in suspension MDCK cells.

Methods: MDCK suspension cells were cultivated in a chemically defined medium (Driving M) in baffled shaker flasks with a working volume of 100 mL (37°C, 5% CO2, 180 rpm). Infection with Influenza A/PR/8/34 (H1N1, RKI) virus was carried out at a concentration of 3-4E+06 cells/mL at an multiplicity of infection (MOI) of 10. Samples for intra- and extracellular protein analysis were taken over a time course of 13 hours post infection (hpi). All samples were heat-inactivated. After TCA precipitation, proteins were reduced and alkylated. Defined amounts (0.2 pmol) of at least two synthetic peptides of each of the four investigated viral proteins were added to each sample before tryptic digest. Mass spectrometric measurements were

Poster presentation

carried out by using a timsTof pro (Bruker) in multiple reaction monitoring mode to imitate a SWATH-MS strategy.

Results: The viral nucleoprotein was already detected at 1 hpi in intracellular samples. Due to the high MOI, it was also detected at 0 hpi in extracellular samples. Next, intracellular hemagglutinin was detected at 3 hpi (4 hpi extracellular), matrix protein 1 at 5 hpi (4 hpi extracellular) and neuraminidase at 5 hpi (5 hpi extracellular). Nucleoprotein also occurred at the highest copy number with 4.3 E+06 copies/cell and 7.7E+12 copies/mL (extracellularly). This was followed by M1 (1.4E+06 copies/cell, 2.2E+12 copies/mL, 13 hpi) and HA (5.5E+05 copies/cell, 11 hpi, 4.1E+12 copies/mL, 13 hpi). The protein with the lowest maximum concentration was NA. With respect to matrix protein 1, relative proportions of all other virus proteins were similar as reported previously using a radioactive labeling method.

Conclusion: For the first time, absolute IAV protein copy numbers were quantified by an MS-based method for infected MDCK cells. The nucleoprotein was the first of the viral proteins to be detected and achieved the highest copy numbers of the four viral proteins examined, intracellularly and extracellularly, respectively. Overall, this study provides important insights into viral protein dynamics during single-round influenza A virus replication.

Fig. 1

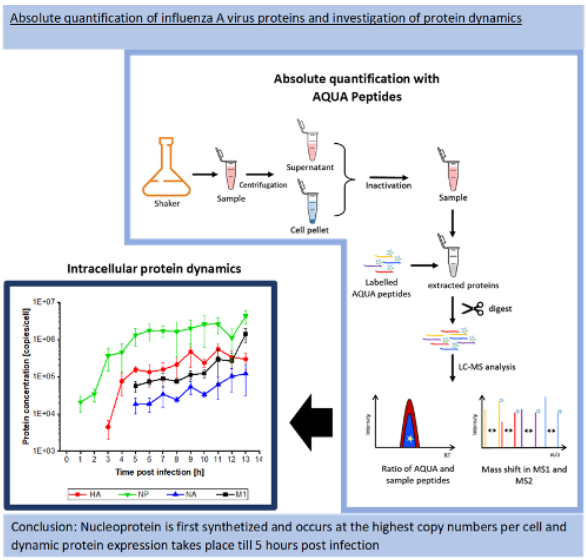
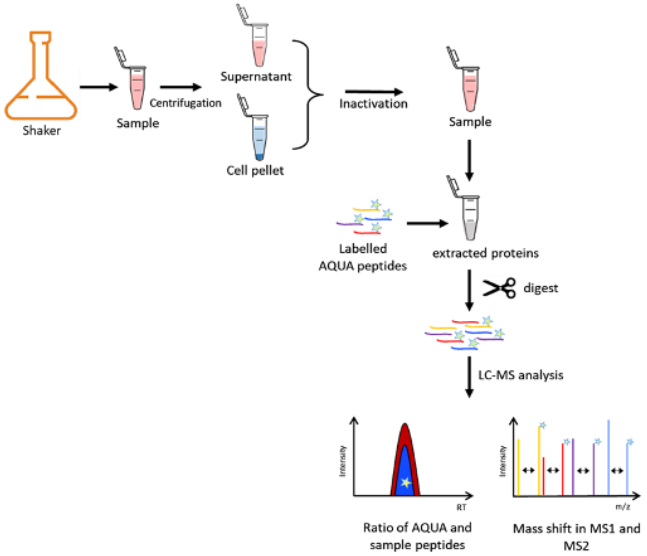


Fig. 2



P17

Proteomics and Mass spectrometry in the early identification of viruses in ovine milk

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Objectives: One of the main causes in economical and livestock losses are viruses. Viruses from a microbiological aspect are difficult to detect and can cause implications with bacteria or other viruses making diagnosis and therapy even harder. When it is a matter of public health or a disease with high morbidity and mortality rate in animals early detection can have a crucial role in the restriction and elimination of the virus. Also, clinical signs may appear in the affected animals many days post infection and incubation period, leading to contamination of the entire flock and the neighboring ones. On the contrary to the latter one, immune response begins shortly after infection. So the concomitant detection of virus and immune response indicators imply that the virus is active and treatment or other precautious measures can be safely applied. In the present study, we scan ovine milk samples originated from Greek farms using High Resolution Mass Spectrometry for virus detection and their link to humoral response.

Methods: For this purpose 20 ovine milk samples taken from various flocks with Chios and Lacaune breeds, across the country and during summer months were

analyzed. The samples arrived frozen in the laboratory and they were treated properly for whey proteins extraction. Afterwards they were further processed according to Anagnostopoulos et al (2016). A tryptic digestion of extracted proteins was necessary in order to generate peptides. LTQ Orbitrap Elite coupled to a Dionex 3000 HPLC system was used to perform the analysis in the digested samples. Finally the results were analyzed in the Proteome Discoverer software, using the *Ovis aries* and *Ruminantia* for ovine milk *.fasta databases.

Results: The proteomic analysis revealed the presence of 16 viruses without counting the different serotypes found within the same virus (Table 1). 10 of them are endemic in Greece and their detection confirmed their presence in the area. The remaining 6 viruses are the most interesting since they are exotic to Greece and Europe. Apart from the viruses" detection, more than 25 proteins linked directly to immune response and symptoms from respiratory and nervous system were also found. The majority of them are involved to pathways of cytokine and immnunoglobulin regulation.

Conclusions: Our findings indicated that LC-MS/MS might be used as a sensitive, rapid tool for early virus detection and as a prediction model about the severity of the symptoms and the animals' immune response. Moreover, a proteomic approach can proved useful for serosurveillance in the Greek and European borders.

Acknowledgements: Supported by the project "FoodOmicsGR-RI Comprehensive Characterization of Foods" (MIS 5029057), under the Action "Reinforcement of the Research and Innovation Infrastructure", funded by the Operational Programme Competitiveness, Entrepreneurship and Innovation (NSRF 2014–2020) and co-financed by Greece and the E.U. (European Regional Development Fund).

Fig. 1

Table 1: Viruses' classification in endemic to Greece or not.

Endemic to Greece		Non-endemic to Greece	
1.	Bluetongue virus (serotypes 1, 10, 11)	1.	Peste des Petits Ruminants virus
2.	Orf virus	2.	Sheeppox virus
3.	Maedi Visna virus	3.	Rift valley fever virus
4.	Ovine astrovirus 1	4.	Middelburg virus
5.	Ovine herpesvirus 2	5.	Crimean-Congo hemorrhagic fever virus
6.	Ovine adenovirus D serotype 7	6.	Foot and mouth disease
7.	Ovis aries papillomavirus 3		
8.	Louping ill virus		
9.	Borna disease virus		
10.	Hunnivirus A		

P18

The Virus-Host Protein Interaction Landscape of Intact Herpesvirus Infected Cell Nuclei Determined by Cross-Linking Mass Spectrometry

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Question: Viruses hijack host cells through host-virus Protein-Protein Interactions (PPIs) that are essential for all stages of the viral life cycle. It is thus necessary to study these virus-host PPIs systematically for a better understanding of viral pathogenicity. Herpesviruses are a class of large enveloped DNA viruses that are characterized by high prevalence in the human population. Their complex life cycle involves the replication and packaging of the viral genetic material inside infected host nuclei. Despite its significant importance, little is known about the molecular and structural underpinnings of host-virus interplay inside this compartment.

Methods: Cross-linking mass spectrometry (XL-MS) has proven to be a powerful technique to directly probe PPIs in a cellular context. Owing to the technical advancements of the technique, in particular the recent developments of enrichable cross-linkers, XL-MS has moved forward to reach in-depth coverage of PPIs from complex biological systems. Here, we combine XL-MS using an enrichable and membrane permeable cross-linker, DSBSO, with crude isolation of nuclei of Herpes Simplex type-1 (HSV-1) or Human Cytomegalovirus (HCMV) infected host cells. In this setup, isolation of intact nuclei enriches nuclear proteins from the large excess of cytosolic host proteins while the enrichable handle on the cross-linker allows further cross-link purification.

Results: Isolation of infected host cell nuclei at early time points of infection also lead to an increase in abundance of viral proteins in the sample by 2 to 3-fold. Thus, it enabled the detection of naturally low-abundant virus-host cross-links. Following this approach, we identified 76 PPIs involving HSV-1 proteins in a single-shot preliminary experiment. We found several previously known PPIs, such as HSV-1 nuclear egress protein 2 (NEC2) binding to Lamin A and

viral immediate early protein ICP22 in association with the member of the FACT complex, SUPT16H. Strikingly, we also observed novel interactions like viral alkaline nuclease UL12 cross-linked to nuclear DNA-sensing IFI16, which has been previously shown to be involved in cytokine signaling as a response to HSV-1 infection.

Conclusion: This approach paves the way to better understand the complex interplay between virus and host and to discover key viral and host players during viral replication in the nucleus. Our ongoing experiments aim to further increase the coverage of cross-links by scaling up the experimental procedure. In a next step, select interactions will be validated using orthogonal assays and investigated for their functional importance.

P19

Unique peptide signatures of Sars-Cov-2 proteome

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In the past years we have introduced two new terms regarding a proteome. ***Core Unique Peptide (CrUP)*** defined as the shortest amino acid sequence appearing solely in one protein of the given proteome and **Composite Unique Peptides** (**CmUP**) which is the concatenation of two or more adjacent CUP distanced at most X aminoacids. Those constitute the ***Uniquome*** of a proteome. For this study we introduce a new use of the ***Uniquome*** by getting the ***CrUP*** of a proteome A vs proteome B. Those are the ***CrUP***s that can be found only on proteome A (can exist on multiple proteins on proteome A but must not exist on proteome B and are names **A/B CrUPs**).

Sars-Cov-2 proteome consists of 16 reviewed proteins plus one currently unreviewed protein annotated on Uniprot database. We analyzed SARS-CoV-2 proteome for unique peptides with a minimum amino acid sequence length (Core Unique Peptides, CrUPs) which does not appear in Human Proteome (Covid/Human-CrUPs). The present study includes the complete registration of the Covid/Human-CrUPs and their metadata analysis. More specifically the size and starting position of Covid/Human-CrUPs were analyzed, while their coverage and density in the Sars-Cov-2 proteome was calculated. Finally, to better understand their function, we studied their previous characteristic in every protein of Sars-Cov-2 separately.

The analysis found that SARS-CoV-2 include 7.503 Covid/Human-CrUPs, with the SPIKE_SARS2 be the protein with the highest density in Covid/Human-CrUPs. Further examination of the identified Covid-CrUPs showed that most of them is comprised of 6 AA. Position analysis revealed that the Covid-CrUPs **appeared with the same ratio in the proteins.** Especially, was observed that spike glycoprotein of Sars-Cov-2 has the biggest uniqueness with 78% density of CrUPs and 100% unique coverage. Extensive analysis of spike proteins in virus variants, indicated that the most important mutation is the P681R in the Delta and Kappa variants, as it produces new Covid/Human-CrUPs around the R685 cleavage site, resulting to the loss of the site immunological capacity, inducing thus the immune escape of the virus, its massive entrance to the host cell, the virus formation and its massive release.

The results of the present study will be useful for the better understanding the virus pathology and for the identification of new immunological targets. To accomplish the aforementioned analysis, our team has developed a novel Software Suite which will be presented as part of this study and will be available for public use as a beta on January 31st 2022.

Fig. 1

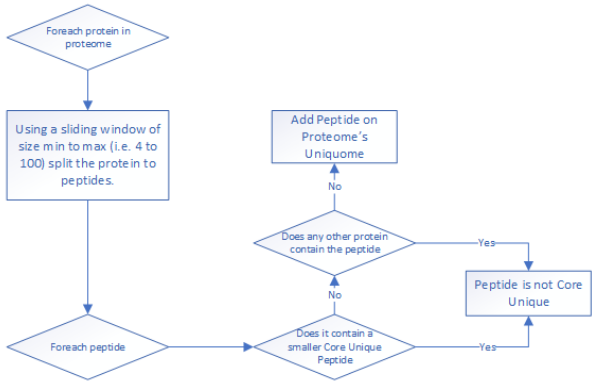

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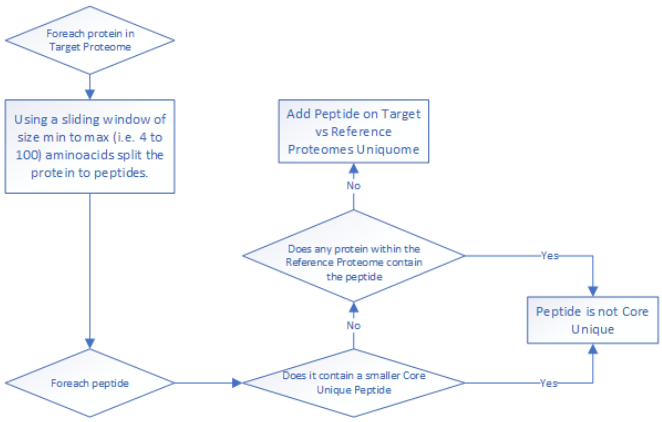
Fig. 2



P20

Comparative analysis of SARS-CoV-2 vector vaccines with special emphasis on proteomics

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Question:

Vaccination is a key factor for the control of the SARS-CoV-2 pandemic. Adenoviral vector-, mRNA-, and antigen-based vaccines are currently in use worldwide. Since February 2021, the rare but severe adverse reaction of vaccine-induced immune thrombotic thrombocytopenia (VITT) has been observed after application of vector-based SARS-CoV-2 vaccines. The adenoviruses used for vaccination are produced in human host cell lines and previous studies have shown that the vaccine ChAdOx1 nCoV-19 still contains a large number of host cell proteins (HCP). In epidemiological studies, the ChAdOx1 nCoV-19 vaccine showed a higher incidence rate of VITT compared to Ad26.COV2.S. Therefore, we comparatively analyzed ChAdOx1 nCov-19 (AstraZeneca) and Ad26.COV2.S (Johnson & Johnson) to reveal differences in the vaccines that might explain the different incidence of VITT.

Methods:

We comprehensively studied ChAdOx1 nCov-19 and Ad26.COV2.S. Both vaccines were comparatively profiled using a DIA-MS approach. Since vector-based SARS-CoV-2 vaccines have been linked to VITT, we also applied transmission electron microscopy, dynamic light-scattering (DLS), single-molecule light microscopy, and a capillary leakage assay to analyse PF4 complex formation or clustering of proteins.

Results:

Our analyses revealed major differences between ChAdOx1 nCoV-19 and Ad26.COV2.S vaccines. ChAdOx1 nCoV-19 vaccine contained a high proportion of HCP (54%), but only a very low level of HCP was detected in Ad26.COV2.S (1.5%). Furthermore, ChAdOx1 nCoV-19 exhibited a high Chymotrypsin-like activity of the proteasome, induced vascular hyperpermeability and formed clusters with PF4.

Conclusion

In summary, we show that process-related impurities in the form of HCP and active proteases are not a general feature of vector-based SARS-CoV-2-vaccines. It has been reported that the incidence rate of VITT is 0.355 per 100.000 for Ad26.COV2.S, compared to 1 per 50,000 - 100,000 people vaccinated with ChAdOx1 nCoV-19. Impurities and the resulting PF4-complex formation might explain the higher incidence rate of VITT for ChAdOx1 nCoV-19. More stringent purification strategy will likely reduce the complex formation of PF4 with vaccine constituents and should be considered in the future.

P21

LC-MS Based Draft Map of the *Arabidopsis thaliana* Nuclear Proteome and Protein Import in Pattern Triggered Immunity

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Objective

Despite its central role as the ark of genetic information and gene expression the plant nucleus is surprisingly understudied. Our objective was to produce a high-quality draft catalog of the *Arabidopsis thaliana* nuclear proteome based on isolation of nuclei and mass spectrometric (MS) measurement of nuclear protein fractions. In addition, we wanted to investigate the quantitative rearrangement of nuclear proteome architecture in pattern triggered immunity (PTI).

Methods

We isolated nuclei from the *Arabidopsis thaliana* dark grown cell culture left untreated and treated with flg22 (22 amino acid N-terminal epitope of flagellin) and nlp20 (characteristic 20 amino acids of virulence factors ethylene-inducing peptide 1 (Nep1)-like proteins), two elicitors of pattern triggered immunity (PTI) in plants, respectively. An LC-MS based discovery proteomics approach was used to measure the nuclear proteome fractions. An enrichment score based on the relative abundance of cytoplasmic, mitochondrial and Golgi markers in the nuclear protein fraction allowed us to curate the nuclear proteome.

Results

We produced a curated high quality *Arabidopsis thaliana* catalog of around 3,000 nuclear proteins under untreated and both PTI conditions. The measurements also covered low abundant proteins including more than 100 transcription factors and transcriptional co-activators. We disclose a list of several hundred potentially dual targeted proteins including proteins not yet found before for further study. Protein import into the nucleus in plant immunity is known. Here we sought to gain a broader impression of this phenomenon employing our proteomics data and found 157 and 73 proteins to possibly be imported into the nucleus upon stimulus with flg22 and nlp20, respectively. Furthermore, the abundance of 93 proteins changed significantly in the nucleus following elicitation of immunity.

Conclusion

These results suggest promiscuous ribosome assembly and a role of prohibitins and cytochrome C in the nucleus in PTI.

Ayash M, Abukhalaf M, Thieme D, Proksch C, Heilmann M, Schattat MH and Hoehenwarter W (2021) LC–MS Based Draft Map of the *Arabidopsis thaliana* Nuclear Proteome and Protein Import in Pattern Triggered Immunity. *Front. Plant Sci*. 12:744103. doi: 10.3389/fpls.2021.744103

P22

Proteomic changes during the germination of different lupin varieties

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Together with common bean, soy and pea, the lupin genus belongs to the legume family. Members of this family benefit from a symbiosis with nitrogen-fixing soil bacteria. Therefore, legumes have increased protein contents and are able grow on nutrient-poor soils without the intensive use of fertilizers. Given the need to reduce greenhouse gases from industrial livestock production, legumes could serve as an alternative protein-source for human nutrition in the future. Unlike soy, lupins are better adapted to the Central European climate and therefore represent a promising source for proteins from plants. However, lupin cultivation may suffer from massive fungal infestation, which recently could be overcome by a new lupin cultivar. To support future research and breeding projects we started to analyze the proteome of lupins in detail. Here, we show first results of proteomic changes during germination of the three most

economically important lupin varieties: *Lupinus albus*, *Lupinus angustifolius* and *Lupinus luteus*. Seeds of these varieties were incubated for 0h, 6h, 12h, 24h, 48h and 72h in moist expanded clay granulate. Proteins were directly extracted, alkylated and digested from freeze-dried material via an adapted single-pot solid-phase-enhanced sample preparations (SP3) on carboxylated and HILIC paramagnetic beads (Mikulasek et al. 2021). Shotgun proteome analysis was then performed using a timsTOF Pro (Bruker) yielding up to 3500 protein species per sample. To obtain information on uncharacterized proteins, the database of *Lupinus albus* (uniprot: UP000464885) was extended by sequence alignments (BLASTn) with genomes from: *Lupinus angustifolius* (uniprot: UP000188354), *Medicago trancatula* (uniprot: UP000002051) and *Arabidopsis thaliana* (TAIR10). This also allowed us to use common bioinformatical tools to determine the subcellular localization (SUBA.live) and gene ontology (mapman.gabipd.org) of identified lupin proteins. Initial results are summarized and discussed.

P23

Proteomic analysis of *Botrytis cinerea* induced noble rot development of Furmint grape berries

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Objective

Botrytis cinerea is a widespread plant pathogen in most regions of the world. As a parasite, *Botrytis cinerea* is responsible for great economic loss in crop production. However, the presence of *Botrytis cinerea* is beneficial in the Tokaj wine region by inducing noble rot. Noble rot is a unique interaction between grape berries and *Botrytis cinerea*, providing chocolate-brown, shriveled berries with exquisite aroma components. Berries transformed by noble rot are the most crucial ingredients of botrytized dessert wines such as the "Aszú" and "Szamorodni" of Tokaj.

In this study, we investigated the process of botrytization by comparing proteomes in four consecutive stages of noble rot.

Methods

Healthy and botrytized *Vitis vinifera* cv. Furmint samples were harvested in vineyard Betsek in Mád. All analyses were performed in five biological replicates, a 4-stage pooled sample was used as a control. After protein extraction from whole deseeded berries, tryptic digestion was performed and the peptides were labeled with tandem mass tag (TMT). Combined samples were fractionated on a high pH fractionation column (Pierce) and subjected to LC-MSMS analysis using nano-UPLC (Waters) coupled to and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Protein identification was based on the CID spectra acquired while SPS MS3 spectra were used for the quantitative analysis. All the analyses were performed with ProteomeDiscoverer 2.4 software.

Results

Botrytis cinerea and *Vitis vinifera* sequences of the Uniprot protein database were used for the SequestHT search. More than 2,000 proteins have been identified, 55 % of which are of plant origin and 45% of which are of fungal origin. As the infection progressed, an increase in the amount of fungal proteins was observed.

Conclusion

Botrytized wines produced in the Tokaj wine region have high cultural and economic importance. The fungal and grapevine proteomes of berries exposed to noble rot have not been studied before. These results provide a valuable insight into the biochemical remodelling of grapevine and *Botrytis cinerea* cells during noble rot. They also help winemakers in producing supreme Tokaj wines.

P24

Phosphoproteomics Analysis of MAMPs Signalling in Plant Immunity

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Vegetable Brassicas are important food crops in the UK but they 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

Poster presentation

Microbe-Associated Molecular Patterns (MAMPs). These MAMP signalling pathways have been widely studied in the model plant Arabidopsis thaliana, but the overlap and differences among these mechanisms remain unclear in crop plants such as Brassica oleracea. 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 hundreds of phosphoproteins who changed their phosphorylation levels in plant defence both of Arabidopsis and Brassica. Black rot is one of the most severe diseases that affects Brassica and caused by the bacterium Xanthomonas campestris pv. campestris (Xcc). Hence, it is better to understand the signalling transduction in Brassica Oleracea when facing with the real disease interaction. This project will draw a latest phosphoproteome map of Brassica Oleracea and offer candidate genes to be used in genetic breeding.

Fig. 1

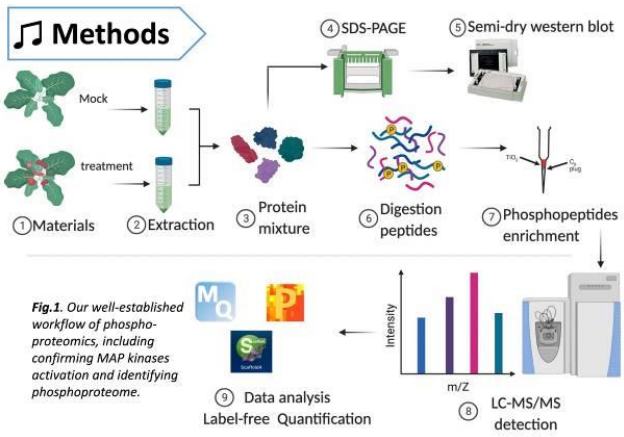
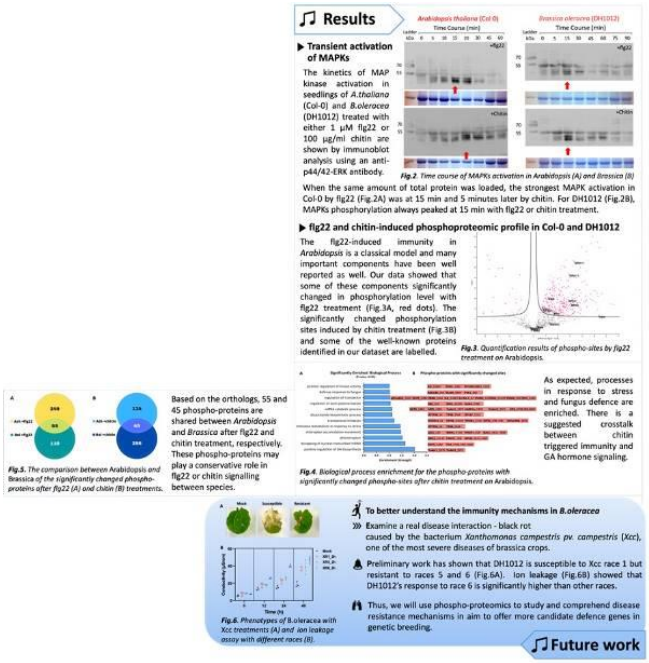


Fig.1. Our well-established workflow of phospho-proteomics, including confirming MAP kinases activation and identifying phosphoproteome.

Fig. 2



P25

Finding novel biocatalysts in environmental samples using functional meta-proteomics

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Biocatalysts are important for a wide range of industries, such as food processing, pharmaceuticals and biofuels. To increase cost-effectiveness and production capacities for these growing sectors, we need novel enzymes with high conversion rates and pH- and temperature stabilities suitable for the application.

Traditionally, new biocatalysts are identified by the creation of metagenomic expression libraries or by searching for similar structural motifs in a sequence database. The former can be time consuming with low success, the latter is a "what you search for, is what you get" approach that makes it difficult to find truly novel enzymes. To overcome these issues, we recently developed a functional metaproteomic method, in which we combine cultivation-independent -omics methodologies with the immediacy of activity screening. It has the potential to discover all enzymes with a given activity expressed in an environmental

community and does not require prior assumptions about the biocatalyst"s structure.

In our approach, proteins are isolated from environmental samples collected in promising habitats. We then utilize two-dimensional polyacrylamide gel electrophoresis to separate the metaproteome. After refolding, activity is directly assessed by in-gel zymography. Active spots can be excised, tryptically digested and analyzed by mass spectrometry. In parallel, we isolate DNA from the very same sample to obtain its metagenome, which is used as reference to reassemble the proteins from the mass spectrometry data. The identified protein can then be obtained recombinantly from a synthetic gene.

We have now applied this method to discover novel amylases, cellulases and hemicellulases, as these enzymes are key to a sustainable utilization of renewable plant-based resources. We extracted protein and DNA from several different habitats, including compost, decaying wood, forest soil and hot springs and were able to identify activity in all of them with our established, robust zymography methods.

Taken together, these results lay the groundwork for a quickly adaptable method. We can now screen multiple enzyme classes in environments that naturally select for desired enzyme properties, making them accessible for industrial applications.

P26

Polysaccharide-degrading potential of microbiomes in agricultural field denitrification beds

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Polysaccharide-degrading strategies have been well-described for various aerobic and anaerobic systems, including the use of carbohydrate-active enzymes (CAZymes), utilization of lytic polysaccharide monooxygenases and other redox enzymes, as well as the use of cellulosomes and polysaccharide utilization loci. However, for denitrifying microorganisms, the lignocellulose degrading strategies are largely unknown. In this project, our aim was to reveal in detail the microbes, enzymes, and metabolic pathways involved in lignocellulose and nitrate-rich habitats in order to infer the degrading strategies at play. We have utilized metagenomics and metaproteomics to study the microbial community within field denitrification beds (FDBs) in Denmark, where nitrate-rich agricultural drainage water is passed through a matrix of willow wood chips. Microbes were extracted from the FDBs and further enriched under denitrifying conditions for one year while regularly monitored for N-gas production and lignocellulose decomposition. Key active microbes and their active CAZymes were identified by integrative meta-omic bioinformatic pipelines and shed new light on the degrading strategies used by these microorganisms.

P27

First insights into the proteome of hemicellulose degrading *Prevotella ruminicola* ATCC 19189

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Objective

The microbial degradation of plant fibers is a key factor of the global carbon cycle and therefore basically affects all living organisms on earth. The whole degradation process of plant cell wall components like cellulose, hemicellulose and other polysaccharides is a complex task carried out by a multitude of different gut microbiome members expressing a great variety of enzymes. Despite its importance, the evolutionary trajectory and the guiding principles of fiber degradation are currently not disclosed in detail. Therefore, the main issue of this project is to unravel the evolutionary, ecological and structural basis of fiber degradation in nature. The aim of this project is to uncover the guiding principles of the establishment of microbial and protein components of natural fiber degradation. For selection of the search strategy, the anaerobic hemicellulolytic strain *Prevotella ruminicola* ATCC 19189 was used. Two different spectral library creation approaches were compared to facilitate the highest number of quantified proteins.

Poster presentation

Methods

P. ruminicola was cultivated with a complex (*Triticum aestivum* fiber) or a simple (fructose) carbon source for 24 h under anaerobic conditions. The cytosolic and enriched secreted proteins were pre-fractionated and tryptic digested prior to the mass spectrometric measurement. The gained data was then investigated with one experimental data-dependent (SEQUEST-based) and one data-independent (Prosit-based) spectral library approach.

Results

More proteins were identified by using the data-independent synthetic spectral library compared to the data-dependent spectral library approach, when evaluating the same samples. More than 1,900 validated proteins were quantified in the analyzed subcellular fractions, which correlates to over 66,5 % of the whole *P. ruminicola* proteome. A slightly higher number of proteins were quantified in the fiber cultured samples compared to fructose cultured samples. These proteins relate to the degradation of hemicellulose (f.e. xylanases, glycoside hydrolases or b-glucosidases) as well as the high number of transporters (f.e. TonB-dependent receptors and Sus-familiy proteins) which indicates that *P. ruminicola* changes its protein repertoire when utilizing the more complex fiber as carbon source.

Conclusions

Here, the first insight into the proteome profiles of *P. ruminicola* in accordance to the used carbon source as well as the applicability of the used data analysis workflow are shown. In the future, the proteome of other dominant members of the gut microbiota will be analyzed with the specifically designed synthetic spectral libraries to gain the highest possible number of correctly assigned proteins. These data will help to better uncover and investigate proteins related to natural fiber degradation.

P28

Combined metagenomics and metaproteomics for analysis of the microbial community in a two-stage biogas plant

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Objective

Anaerobic digesters are important renewable energy sources in which complex microbial communities degrade biomass into biogas (CH4 and CO2). Biogas is converted into electricity and heat by combined heat and power plants. A fundamental understanding of the taxonomic and functional composition of microbial communities in biogas plants is important to increase productivity and yield. The biogas process consists of four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Due to the different environmental requirements of the microbes involved, the process can be separated in the hydrolysis fermenter optimized for hydrolysis and acidogenesis and the main fermenter optimized for acetogenesis and methanogenesis. In this study, metaproteomics of an agricultural two-stage biogas plant was performed to investigate the adaptation of microbial communities in hydrolysis and main fermenters on a taxonomic and functional level and analyze degradation performance.

Methods

Hydrolysis and main fermenter were sampled monthly for one year. For one sample the metagenome was sequenced. For all samples, proteins were extracted

using phenol and measured with liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS). The resulting spectra were searched with the MetaProteomAnalyzer (MPA) against the annotated and binned metagenome. Unique peptides were used for taxonomic and functional analysis. Additionally, the MPA_Pathway_Tool was used to map the microbiome data to the top 50 common metabolic pathways. Finally, the results of the metagenomic data were compared with the metaproteomic data.

Results

The technical and chemical parameters indicated a stable operation of the main fermenter, but varying conditions in the hydrolysis fermenter over the sampling period. Accordingly, microbiome composition was more variable in the hydrolysis fermenter than in the main fermenter.

Metaproteomics analysis resulted in 2,374,049 identified spectra and 90,606 identified proteins. About 75% of all identified spectra could be assigned to the 262 Metagenome-Assembled Genomes (MAGs). Degradation of simple carbohydrates and primary fermentation took place mainly in the hydrolysis fermenter, whereas degradation of complex sugars, secondary fermentation, and methanogenesis was at a high level in the main fermenter. Furthermore, metaproteomics revealed that not all microbes utilize their entire genetic potential (e.g. cellulose degradation). In addition, a seasonal increase in phage proteins was detected in the hydrolysis fermenter.

Conclusions

The combined metagenomic and metaproteomic analysis of a two-stage biogas plant showed the adaption of the microbial communities due to the different conditions in the hydrolysis and main fermenter. The fact that hydrolysis of complex sugars like cellulose was preferred in the main fermenter questions whether a two-step biogas plant including a separate hydrolysis fermenter increases the biogas yield.

P29

Metaproteomic investigation of marine sediment to evaluate the metabolic potential for hydrocarbon degradation

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Question: The project PROSPECTOMICS aims to develop a fundamentally new approach for oil and gas exploration in marine environments based on molecular biological methods in order to minimize the environmental impact compared to currently used geological and geophysical prospection techniques. Seepage from submarine oil and gas reservoirs does not always reach the sea floor but can induce a shift in microbial community composition and its metabolic activity profile in the subsurface sediment. The microbial footprint and biochemical properties of sediments above hydrocarbon deposits will be compared to sediments in areas without the occurrence of oil or gas, using biogeochemical approaches as well as "omics" technologies like metagenomics, metatranscriptomics and metaproteomics.

Currently, the different groups involved in the project are focusing in the extraction of biomolecules like DNA, RNA and protein from marine sediment. Obstacles in protein extraction from these environmental samples are caused by the composition of the sediment. Sediment particles can bind proteins and trap them. Additionally, humic substances are coextracted with proteins and interfere with colorimetric assays for determination of protein concentration as well as disturb mass spectrometric measurements.

Methods: A standardized method for protein extraction from marine sediment is not available yet. Shelf sediment from the Barents Sea was used for optimizing protein extraction methods. Therefore, different published extraction procedures were tested

including cell disruption with heat and/or ultra-sonication in several extraction buffers as well as enrichment of proteins with tricarboxylic acid precipitation or binding on StrataClean beads. Finally, the extracted proteins were processed with an SDS-PAGE and tryptic digested. Resulting peptides were eluted from the gel and desalted by C18 ZipTips prior to mass spectrometric measurement. The number of recorded MS spectra as well as the number of identified proteins were used as indicator to compare the methods regarding their efficiency to extract proteins from this challenging environment. Since no sample-specific database was available, a protein database containing marine sediment organisms was constructed.

Results: In a first attempt, about 50 proteins could be identified with a minimum of two peptides by using a combination of heating and ultra-sonication of the samples prior to protein enrichment using StrataClean beads. Using the different extraction approaches, up to 150 proteins could be identified with at least one peptide. The low number of identified proteins indicates their low concentration in the extracts.

Conclusion: To conclude, the tested extraction methods can be used for protein extraction from marine sediments but have to be further optimized in order to increase the number of identifiable proteins. Furthermore, sample specific metagenomes are expected to increase protein identification rates.

P30

Analysis of the impact of perfluoroalkyl substance exposure on a simplified human intestinal microbiota using proteomics and metabolomics

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Objective: The rich collection of intestinal microbes, collectively termed the gut microbiome, is an indispensable part of the human host and is modulated throughout life. Ingested food components and environmental pollutants have the potential to disturb a balanced microbiome resulting in adverse outcomes for host health. Per- and polyfluoroalkyl substances (PFAS) are a class of human-made chemicals that are broadly applied as surfactants in coatings, inks and fire-fighting foams. They display long half-lives and are hence ubiquitously present in environment and human body. As uptake via the diet poses the major exposure route, we aimed to broaden the knowledge on the effect of PFAS on the human gut microbiota.

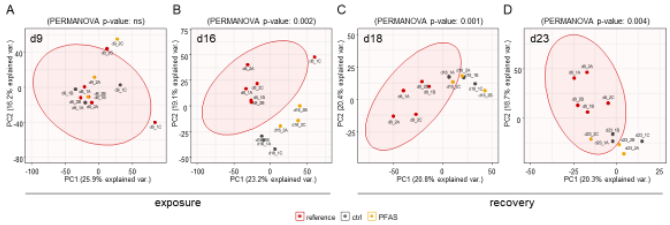
Methods: For this purpose, we investigated intestinal single strains and the simplified human intestinal microbiota SIHUMIx grown as in vitro continuous culture during exposure to a set of PFAS with different fluorinated chain length and functional head groups (Perfluorosulfonic acid (PFOS), perfluorooctanoic (PFOA)/-hexanoic (PFHxA)/-butanoic (PFBA) acid). Functional analysis was carried out using global (meta-)proteomics and targeted metabolomics of short chain fatty acids. Membrane integrity of Bacteroides thetaiotaomicron was analyzed using fatty acid methyl ester profiles.

Results: We demonstrated an increasing toxicity of PFAS with increasing carbon chain length and altered membrane fatty acid compositions in exposed B. thetaiotaomicron cells. Susceptibility to PFAS was decisively determined by the bacterial membrane gram-type, with gram-positive bacteria being more negatively affected. Reversible taxonomic and functional alterations induced by PFAS exposure were observed in SIHUMIx after long-term toxicant exposure of eight consecutive days. Among them general stress responsive proteins and altered transmembrane transport. With two affected core functions of the gut microbiome, namely carbohydrate binding and uptake as well as biosynthesis and transport of vitamins and cofactors such as vitamin B1, B5 and B7, the PFAS induced alterations are potentially directly linked to host health.

Conclusion: In summary, we were able to reveal mechanistic insights into PFAS-induced effects on a simplified human gut microbiome, which are highly relevant to understand their impact on human health.

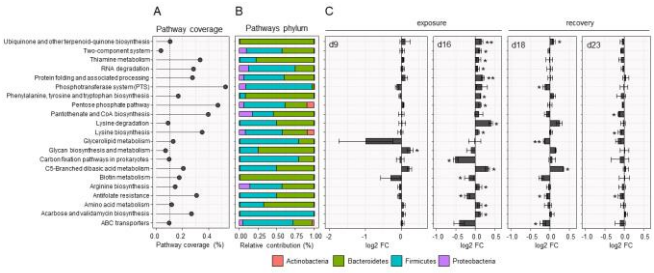
Poster presentation

Fig. 1



Attachment 1: SIHUMIx metaproteome development in response to long-term PFAS exposure. PCA plots in the exposure phase at day 9 (**A**) and day 16 (**B**) and in the recovery phase at day 18 (**C**) and day 23 (**D**) were calculated on basis of the metaproteomic results. Given as reference state (red dots and ellipses) in all plots is the community at the end of the stabilization phase at day 8 (n = 6). Control reactors are marked in black and labelled with day and the respective reactor identifier (1A, B, C). PFAS treated reactors in yellow (2A, B, C) (active PFAS treatment in exposure phase only). Distinct treatment group separation occured at the end of exposure phase and closed up again during treatment recovery.

Fig. 2



Attachment 2: Effects of PFAS exposure on SIHUMIx metabolic pathways. Pathways were selected on basis of their significantly different abundance between control and PFAS treated reactors. (**A**) Pathway coverage was calculated as number of pathway annotated proteins in the filtered subset divided by the number of total pathway annotated proteins in the KEGG database. The vertical dotted line indicates the set threshold of 10 % pathway coverage. (**B**) Relative contribution of the four phyla present in SIHUMIx to the pathway annotations. Actinobacteria: *Bifidobacterium longum*; Bacteroidetes: *Bacteroides thetaiotaomicron*; Firmicutes: *Anaerostipes caccae*, *Clostridium butyricum*, *Blautia producta*, *Eryspelotrichostridium ramosum*, *Lactobacillus plantarum*; Proteobacteria: *Escherichia coli*. (**C**) log_2 fold changes of selected pathways in exposure (d9, 16) and recovery (d18, 23) phase. A students *t*-test was performed and significances are indicated as following: * $p \leq 0.05$, ** $p \leq 0.01$.

P31

Rapid quantitative characterization of microbial communities using ultrafast MS/MS-free proteomics

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Objective: Metaproteomics is an emerging area to study biological communities, the functioning and role of each community member, as well as changes induced by the environment. Liquid chromatography/tandem mass spectrometry is a method of choice in metaproteomics, yet its current implementation is instrument time consuming, thus making this analysis inefficient. The main objective of this study is to develop an ultra-fast and cost-effective method based on high-resolution mass spectrometry for metaproteomic research.

Methods: Data collected for 19 bacterial strains were used to develop a two-step search strategy for identifying microorganism species using ms1searchpy engine of the MS/MS-free method of proteome analysis DirectMS1 (PMID: 33720732). Peptide feature detection was performed using Biosaur (PMID: 33450063). Strains were matched against bacterial TrEMBL and customized proteogenomic databases. Target-decoy approach was used to control protein FDR. To develop an algorithm for quantitative determination of microbial community composition and examine the method's sensitivity, the model microbiomes were assembled. Tryptic peptides of *Rhodococcus opacus 1CP*, *Rhodococcus erythropolis X5*, *Priestia aryabhattai 25*, *Gordonia alkanivorans 135* and *Gordonia amicalis* were mixed at different ratios and analyzed using 5-min LC gradient and MS1-only mode for spectra acquisition. Diffacto (PMID: 28302922) was used for statistical assessment of changes in microbiome compositions.

Results: Two-step search to identify bacterial species from LC-MS1 data against combined bacterial databases demonstrated correct identification of 84% species. The search algorithm correctly recognized the bacterial species and differentiated the strains of the same phylogenetic group when the complete and non-redundant strain databases are included in the search space. We demonstrated the feasibility of our method to obtain quantitative ratios between mixed strains and explored its sensitivity limits. Specifically, the observation of at least 30% of unique peptides per strain resulted in correct recovering the quantitative ratios.

Conclusion: The suggested analytical pipeline based on ultrafast LC-MS1 proteomics has demonstrated its high utility for identification and quantitative characterization of complex microbial communities and can be successfully applied in metaproteomic studies.

Funding: Bacteria strains, genome sequencing and annotation: RFBR No.19-54-80003. Metaproteome analysis: RSF No.20-14-00229.

P32

Co-culturing simplified human Intestinal microbiota (SIHUMIx) strains with *Eggerthella lenta* DSM 2243 increases bile acid tolerance

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Objective: Cross-communication between host and the gut has been recognized for its immense influences in disease and health. The gut-liver axis has been shown to influence human physiology in *via* chemical modification of primary bile acids produced by the host, resulting in secondary bile acids. Both primary and secondary bile acids are known to modulate lipid and glucose metabolism as well as having antimicrobial properties, thus affecting the composition of the microbiome. Specifically, keto and iso forms of bile acids are emerging as molecules of interest in gut-liver cross communication, since their effects are mostly unknown both in the gut and on the host, as well as which species are capable of it. Understanding the effects of keto/iso bile acids and the bacteria capable of creating them, will provide vital information on cross-communication between the gut and the liver, in turn improving our understanding of gut health and disease.

Methods: Strains from the SIHUMIx model system were cultured *in vitro* under anaerobic conditions. Strains were grown either alone or co-cultured with *Eggerthella lenta* (*E.lenta*) and stressed with a concentration range of cholic acid (CA) or deoxycholic acid (DCA), ranging from 10 to 600 µM for 6 hours. Metaproteomic and metabolomic analysis were conducted to gain functional insights into the specific effects of bile acids, and the significance of a bile acid modifying bacteria.

Results: We report here that strains grown alone had a significant decrease in growth when stressed with 600µM DCA compared to non-stressed cultures, where no significant decrease was seen with CA. Furthermore, when strains were co-cultured with *E.lenta* inhibition of growth by DCA was diminished. Gram negative strains were more inhibited by DCA than gram positive strains. In the ongoing proteomic analysis, and we expect to see that co-culturing with *E.lenta* increases bile acid tolerance by expressing hydroxysteroid dehydrogenases (HSDs) enzymes that can hence modify bile acids and decrease their toxicity towards bacteria. Furthermore, metabolomic measurements are also ongoing, and we expect to see decreases in the dosed bile acids, and potentially detect keto / iso forms of them. Untargeted metabolomic measurements are also being conducted.

Conclusion: DCA decreases the growth of bacteria, in particular those incapable of expressing HSDs required for modifying bile acids. This decrease could be inhibited *via* co-culturing with *E.lenta* when strains were capable of growing together. Several strains were capable of modifying bile acids, but *E.lenta* in particular. These findings indicate that bacteria capable of modifying bile acids are crucial for mediating a normal composition and function of the gut microbiome.

P33

A combined minion-metaproteomics approach for the characterization of cow milk microbiome and resistome in a one-health perspective

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Question

Dairy cow mastitis (CM) is the cause of a great financial loss for all the animal production industry. The average cost of a single case of CM is around € 200. Its diagnosis increases the costs as well as the requirement of a more challenging treatment with antibiotics. Antimicrobial resistance (AMR) is a natural process that causes the selection and dissemination of antimicrobial resistance genes (ARGs) and is mainly reinforced by the overuse or inappropriate use of antimicrobials., Moreover ARG transmission between different ecosystems in a One-Health perspective is only partially understood. In this context, the study of resistome, pangenome and metaproteome can be achieved using modern omics tools like whole genome sequencing, metagenomics, and proteomics [1]. The main aim of this work is to analyze through a multi-

omic approach both the bacterial and cellular compartment of cow milk to gain informations about the whole bacterial composition and the effects of antibiotic residues on the milk microbiome for a better comprehension of the dynamics of AMR flux between the One-Health ecosystems.

Methods

Through the metagenomics approach (using third-generation sequencing technology), the microbiome of milk samples classified for several variables has been analyzed to recover the taxonomical profile of each sample. NGS-specific bioinformatics pipelines have been applied (EPI2ME and METONTTIME) to obtain 16S taxonomic information and non-phylogenetic diversity metrics between samples. The metaproteomic approach has been focused on the detection of key ARGs expressed in milk microbiome. Mass spectrometry-based analysis using specific databases (e.g., CARD) and pipelines (METALAB2) allowed the monitoring of the relative abundance of ARG-related proteins among each sample analyzed and the bacterial species associated with the AMR dissemination.

Results and Conclusions

To the best of our knowledge, this method may allow the characterization of key ARGs expressed in cow milk microbiome and provide a useful tool of practical importance for the detection of the indirect presence of antimicrobial contamination in the environment to help a better understanding of the ARGs flow between different One-Health ecosystems.

[1]. Piras C, Greco V, Gugliandolo E, Soggiu A, Tilocca B, Bonizzi L, Zecconi A, Cramer R, Britti D, Urbani A, Roncada P. Raw Cow Milk Bacterial Consortium as Bioindicator of Circulating Anti-Microbial Resistance (AMR). Animals (Basel). 2020 Dec 11;10(12):2378. doi: 10.3390/ani10122378. PMID: 33322611; PMCID: PMC7763537.

Acknowledgements: work supported by PRIN 2017 "SAFE MILK: OMICS SCIENCE FOR MILK SAFETY AND QUALITY" (grant No. 2017MZ5KWM)

P34

Proteomics to gain insights into the mechanisms of direct and indirect drug-induced thyroid toxicity

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Objective

The thyroid gland regulates various physiological mechanisms in mammals/humans, such as individual development, cell proliferation and differentiation. Thus, disorders can lead to diseases. Several clinical agents have been described to cause drug-induced thyroid toxicity (DITT) by directly interfering with the synthesis, transport and metabolism of thyroid hormones, or by altering the synthesis and secretion of thyrotropin. However, the underlying mechanism of DITT is still not fully understood, thus hindering the risk assessment of e.g., novel drugs or chemicals.

Methods

We applied an untargeted proteomics study to investigate DITT in rodents as part of a multi-omics study in the framework of the CEFIC-funded LRI-C5 project "XomeTox – evaluating multi-omics integration for assessing rodent thyroid toxicity". Male Wistar rats were exposed to a direct (6-propyl-2-thiouracil, PTU) and an indirect (phenytoin) thyroid toxicant, and proteomics was applied to thyroid and liver tissues. Here a low and a high dose were tested, and samples were analyzed at three time points, including one after a recovery phase. Drug-induced significant changes were investigated compared to untreated controls, followed by KEGG enrichment based on the significantly altered proteins (SAPs).

Results

We found that the use of phenytoin did not affect the thyroid, whereas slight time-dependent effects were

observed at the high dose in liver. Most affected proteins were associated with xenobiotics metabolism via cytochrome P450. Remarkably, after the recovery phase, no further changes were evident, indicating full recovery. In contrast, after PTU treatment, stronger time-dependent effects were observed in liver after exposure to the high dose, which were again associated with xenobiotics metabolism via cytochrome P450 but also with carbon and amino acid metabolism. These effects were mitigated after the recovery phase. In contrast, in thyroid samples after PTU treatment, full recovery was achieved only with the low dose. SAPs in PTU-treated thyroid tissue were mainly involved in carbon and amino acid metabolism, thermogenesis, oxidative phosphorylation and lysosomal processes. The latter remained significantly enriched after the recovery phase. These results suggest the PTU effects to be more severe and complex in thyroid than in liver.

In addition, weighted gene correlation network analysis was applied to identify key drivers for DITT. Thus, members of the P450 family involved in hepatic metabolism and detoxification, such as CYP2B1 and CYP2C70, were found to be associated with drug-induced hepatotoxicity and DITT. And ATP1B1 was found highly connected to DITT, which was upregulated upon treatment with PTU and not changed after the recovery phase.

Conclusion

With this study, we gained mechanistic insights in direct and indirect DITT and identified key drivers, which allow insights into the modes of action, thus potentially facilitating future risk assessment.

P35

A MALDI-TOF assay identifies nilotinib as an inhibitor of inflammation in acute myeloid leukemia

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Inflammatory responses are important in cancer, particularly in the context of monocyte-rich aggressive myeloid neoplasm. We developed a label-free cellular phenotypic drug discovery assay to identify anti-inflammatory drugs in human monocytes derived from acute myeloid leukemia (AML), by tracking several biological features ionizing from only 2,500 cells using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. A proof-of-concept screen showed that the BCR-ABL inhibitor nilotinib, but not the structurally similar imatinib, blocks inflammatory responses. In order to identify the cellular (off-)targets of nilotinib, we performed thermal proteome profiling (TPP) using TMTpro 16-plex. Unlike imatinib, nilotinib inhibits the MAPK14 (p38 alpha)-MK2/3 signaling axis. This inhibition suppressed the expression of inflammatory cytokines, cell adhesion and innate immunity markers in activated human monocytes derived from AML. Thus, our study provides a tool for the discovery of new anti-inflammatory drugs, which could contribute to the treatment of inflammation in myeloid neoplasms and other diseases.

P36

Finely tuned mass spectrometry-based workflows for trace-level Host Cell Protein impurities characterization in biotherapeutics

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Question:

Removal of host cell proteins (HCP), deriving from the cell expression system, during therapeutic recombinant proteins production is considered as a critical concern, since their presence in final drug products can jeopardize the drug efficacy and threaten patient safety (Bracewell D.G. *et al.,* 2015). Therefore, the mass spectrometry (MS) and proteomics toolbox is establishing itself as a promising method for precise and accurate quantification of HCP impurities in final drug products.

Methods:

LC-MS/MS analyses were performed on an Orbitrap Eclipse™ Tribrid™ mass spectrometer (Thermo Scientific™). The contribution of data-independent acquisition (DIA) was evaluated to appraise improvements in HCP identification and quantification in the NIST Monoclonal Antibody Reference and several commercial Drug Products. Additionally, the benefit of employing high-field asymmetric waveform ion mobility spectrometry (FAIMS) with multiple compensation voltages (CV) in data-dependent

acquisition (DDA) and DIA modes was assessed. Homemade sample-specific spectral libraries were generated in order to perform DIA data extraction in a peptide-centric manner and its performances were compared to a spectrum-centric approach.

Results:

MS front-end FAIMS separation and a range of acquisition modes (including DDA and DIA methods) available on the Orbitrap Eclipse™ Tribrid™ were evaluated for their potential to improve the characterization and quantification of ppm-level host cell proteins in the NIST mAb standard and several purified final drug products. HCP-Profiler standard beads (Anaquant) were spiked into all samples to use internal calibration curves for improved quantification combined with a Top 3 strategy (Silva J.C. *et al.*, 2006) – based on the MS signal response of the three most intense tryptic peptides of a protein. In comparison to DDA, DIA-based quantification demonstrates higher numbers of detected peptides per protein and more than 30% gain in total quantified HCP amounts in the samples. Additionally, the spectrum-centric DIA extraction strategy showed a 25% raise in the number of quantified peptides in contrast to the spectral library peptide-centric approach, while being straightforward and easy to execute for DIA experiments. Lastly, the implementation of an additional ion mobility separation using a FAIMS device shows benefits to dig deeper into trace-level HCP, by doubling the number of quantified proteins and leading to higher global amount of impurities.

Conclusion:

This study demonstrated the potential of using quantitative DDA and DIA approaches, with internal calibration curves and Top3 quantification, for accurate identification and quantification of HCP. Moreover, the implementation of the FAIMS technology enabled deeper characterization of trace-level protein impurities in final drug products.

P37

Enhanced selectivity and sensitivity for peptide quantification in a complex matrix using high – resolution LCresolution LC--MS/MS workflowMS/MS workflow

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Given the increased focus on producing new protein and peptide therapeutics, there is a resulting demand for highly sensitive and robust quantitative bioanalytical techniques to ensure proper testing for their safety and highly sensitive and robust quantitative bioanalytical techniques to ensure proper testing for their safety and efficacy. Bioanalysis of peptide therapeutics is often faced with analytical challenges such as inadequate efficacy. Bioanalysis of peptide therapeutics is often faced with analytical challenges such as inadequate sensitivity and complexity of the matrix resulting in poor selectivity. Highsensitivity and complexity of the matrix resulting in poor selectivity. High--resolution accurate mass spectrometry resolution accurate mass spectrometry (HRAMS) has been increasingly adopted in bioanalytical workflows as it provides high selectivity with narrow (HRAMS) has been increasingly adopted in bioanalytical workflows as it provides high selectivity with narrow mass extraction windows. As part of this work, evaluation of enhanced duty cycle by a novel ion beam to timemass extraction windows. As part of this work, evaluation of enhanced duty cycle by a novel ion beam to time--ofof--flight (TOF) pulser efficiency was performed. The new quantitative enhancements were evaluated for peptide flight (TOF) pulser efficiency was performed. The new quantitative enhancements were evaluated for peptide quantification in a complex biological matrix.quantification in a complex biological matrix.

.An average of 5An average of 5--fold in LLOQ improvement was achieved for peptide quantification in this sample set using the Zeno trap, which fold in LLOQ improvement was achieved for peptide quantification in this sample set using the Zeno trap, which enhances the duty cycle through the accumulation of ions during each TOF pulseenhances the duty cycle through the accumulation of ions during each TOF pulse

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.A highly accurate and reproducible quantitative workflow for peptides was demonstrated using the A highly accurate and reproducible quantitative workflow for peptides was demonstrated using the

ZenoTOFZenoTOF7600 system7600 system
.
.Greater selectivity was reached between target peptides and matrixGreater selectivity was reached between target peptides and matrix--related components with the higher mass resolution offered byrelated components with the higher mass resolution offered bythe ZenoTOFZenoTOF7600 system7600 system

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.Automated and accurate peak integration was easily attainable on the Automated and accurate peak integration was easily attainable on the ZenoTOFZenoTOF7600 system, with greater mass resolution ensuring 7600 system, with greater mass resolution ensuring overall data integrityoverall data integrity

P38

Species-Specific Impact of Fusarium Infection on the Shoot Proteome of Asparagus

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Infection with soil-borne pathogens have considerable detrimental effects on asparagus (*Asparagus officinalis*) growth and production, notably caused by the Fusarium species *F. oxysporum f.sp. asparagi*, *F. proliferatum* and *F. redolens*. Pot experiments showed a significant negative impact of those three Fusarium species on the root characteristics total root length, volume, and surface area, with *F. redolens* isolates causing the strongest effects. Upon infection, the pathogen spreads systemically inside the plant and, hence, fungal DNA was detected in the basal part of the plant stem.

In the present study, we compared the proteome of infected basal stems with that of control plants to get insight into the species-specific impact of Fusarium infection. Three independent infection experiments were performed with 4 months old plants being inoculated with the respective Fusarium strains and harvested 8 weeks post inoculation. For each experiment, three replicates of plants were randomized, each replicate consisting of three plants (n=27 per one treatment, treatments: control, four isolates of *F. oxysporum f.sp. asparagi*, three isolates of *F. proliferatum* and two isolates of *F. redolens*). Protein extracts of single plant samples were digested with trypsin, desalted and analysed using LC-MS/MS.

On average, 4500 proteins were detected in each sample. When compared to the control samples, on average 200 proteins were significantly enriched and 400 proteins were significantly down-regulated upon the infection. For the display of species-specific overlap in the proteome response, we used Venn diagrams and classification of differentially abundant proteins according to MapMan bins. First results of these classifications from the comparison of infected and non-infected plants are presented. Using such approaches we will gain insight into the diversity in the interaction process of asparagus with different species of Fusarium for developing sustainable measures for disease control and to improve plant health.

P39

Reduced level of tear antimicrobial and immunomodulatory proteins as a possible reason for higher ocular infections in patients with diabetes mellitus

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Objective: Diabetes mellitus is one of the most common metabolic disorders and a risk factor for bacterial ocular infections. Our aim was to examine the antibacterial activity of tears from patients with diabetes mellitus with and without diabetic retinopathy and to link this activity to the level of tear proteins.

Methods: Non-stimulated basal tears were collected from 39 eyes of 35 subjects. The antibacterial activity of tear pools was tested against pathogenic *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 26922 and *Pseudomonas aeruginosa* ATCC 27853 strains. The levels of 10 antimicrobial and

immunomodulatory proteins were analyzed in the individual tear samples of the studied groups by SRM-based targeted mass spectrometry analysis.

Results: Disease stage-specific antimicrobial effect was observed in case of *Staphylococcus aureus* ATCC 29213 strain, and a non-disease specific inhibitory effect was observed in case of *Pseudomonas aeruginosa* ATCC 27853 strain. Changes in the levels of the studied antimicrobial and immunomodulatory proteins in the tears of the studied groups were also observed.

Conclusion: The higher ocular infection rate observed in diabetic patients may be the consequence of the decreased antimicrobial activity of tears possibly caused by the changes in the levels of antimicrobial and immunomodulatory proteins.

Acknowledgement: This research was funded by TÁMOP 4.2.2.A-11/1/KONV-2012-0045 and GINOP-2.3.3-15-2016-00020 funding.

P40

N-terminomic analysis in *Staphylococcus aureus* enables identification of not annotated N-termini and proteoforms

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Objectives:

Identification of proteins by bottom-up proteomics is based on the analysis of proteolytic peptides using mass spectrometry and protein databases usually obtained from automatic genome annotations. N-terminal peptides of proteins are frequently missing in these analyses. Reasons are as follows: (i) post-translational proteolytic cleavage of proteins that generates novel N-termini, (ii) several post-translational modifications, (iii) proteolytic N-terminal peptides that do not meet MS-requirements and/or (iv) misannotations of translational start sites. Hence, N-terminomics approaches in combination with ribosomal profiling have been widely used to understand proteolytic processing of proteins and for improving genome annotation.

Methods:

For identification of N-terminal peptides in *S. aureus* Newman, we applied an N-terminomics approach, which was based on N-acetylation of protein N-termini and their enrichment (McDonald and Beynon, 2006). In addition, an actinonin based proteogenomics approach was implemented to verify native protein N-termini. The obtained MS data were analyzed by using a customized N-terminal proteoform database (NTDB), which combined the existing NCBI FASTA file of *S. aureus* Newman with additional evidence-based predicted protein sequences and possible N-terminal elongated and truncated sequence variants of each protein. For peptide identification, only high quality peptide spectrum matches were considered.

Results:

In this way, we were able to identify not only annotated but also not annotated protein N-termini, which revealed among other things protein variants with differing N-termini.

McDonald, L. and Beynon, R. J. (2006). Positional proteomics: preparation of amino-terminal peptides as a strategy for proteome simplification and characterization. Nature protocols, 1(4), 1790–1798. https://doi.org/10.1038/nprot.2006.317

The authors declare no conflict of interest.

P41

Identification and analysis of exogenous peptides in human blood plasma. Search for potential agents of interaction between the intestinal microbiota and the human body

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Symbiotic interaction between the human body and its microbiota is an important issue of modern biomedicine and personalized medicine. However, little is known on molecular mechanisms of that relationship. Bearing in mind the ubiquitous participation of peptides in biomolecular interactions and regulatory processes we attempted direct search of blood peptides originated from microbial proteins. LC-MS/MS analysis was carried out of blood serum and plasma samples taken from 20 healthy donors on Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass-spectrometer. Sample preparation was carried out based on our previously developed method of peptide desorption from the surface of major blood plasma proteins followed by standard chromatographic steps. Mascot and X! Tandem search engines were used for peptide identification. Human protein sequences were taken from UniProt Knowledgebase and sequences of human microbiota proteins – from NIH Human Microbiome Project.

As a result, out of 13,625 identified peptides 912 were unique fragments of microbial precursors, which is about 6.69% of the total amount of detected bloodstream peptides. In 30 cases peptide identification was confirmed by mass-spectral study of individual synthetic samples. Absolute quantification by the mass-spectrometric method of multiple reaction monitoring (MRM) confirmed the presence of bacterial peptides in plasma and serum in the range of approximately 0.1 nM to 1 uM, which is comparable to physiologically significant hormone concentrations in human blood in normal conditions. The abundance of microbiota peptides reaches its maximum 5h after a meal. Most of the peptides correlate with the bacterial composition of the small intestine and are likely obtained by hydrolysis of membrane proteins with trypsin, chymotrypsin and pepsin — the main proteases of the gastrointestinal tract.

The isolated fraction of peripheral blood mononuclear cells showed increase secretion of proinflammatory cytokines, colony stimulating factors and chemoattractants as the response to the addition of some of the identified microbiotic peptides. Such peptides — identified both in the plasma and in the serum — have properties of resistance to fibrinolysis, since the pool of peptides is preserved after passing through the digestive tract. The physicochemical properties of the identified bacterial peptides are consistent with those required for the selective permeability of mucosal barriers. Our approach to the identification of microbiota peptides in the blood serum and plasma may be useful for determining the microbiota composition of hard-to-reach intestinal areas, such as the small intestine, and for monitoring the permeability of the intestinal mucosal barrier. The microbiota peptides that we were able to identify in the plasma and serum of healthy persons can be used for further research on host–pathogen interactions.

This work was supported by the Russian Science Foundation project no. 20-15-00400.

P42

Physiology of a *trans*-translation deficient *Bacillus subtilis* mutant – a comparative proteomics study

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Mechanisms to release stalled ribosomes are essential for the growth and survival of bacteria [1]. *trans*-Translation is the most effective and widespread of such mechanisms. Previous studies show that *trans*-translation plays an important role under stress conditions such as high temperature and exposure to antibiotics [2,3]. Although *trans*-translation can be crucial in some bacteria, *Bacillus subtilis* has two alternative rescue mechanisms, which rely on the

proteins BrfA and RqcH, that ensure its viability when *trans*-translation is deficient [4]. *Objective:* We sought a deeper understanding of the impact of *trans*-translation deficiency on *B. subtilis* physiology. *Methods:* a quantitative Label-free LC-MS/MS analysis was performed to compare the proteomes of the *trans*-translation-deficient Δ*ssrA* mutant to that of *B. subtilis* 168 during the log phase. *Results:* In chemical defined medium, the *B. subtilis* Δ*ssrA* mutant has a growth rate 33% lower than *B. subtilis* 168. Voronoi treemaps show a higher representation of ribosomal proteins in the mutant (30% compared to 22% in *B. subtilis* 168) and a 100% over-representation of chemotaxis and motility-related proteins. Furthermore, in the mutant, proteins from amino acid metabolic pathways make up 4% less of the total protein. The proteomic comparison also revealed 34 overrepresented proteins in the mutant, of which eight are related to chemotaxis. Sixty proteins were underrepresented, most of which are related to amino acid biosynthesis and cell wall organization. *Conclusion:* Based on the proteome profile, we hypothesize that in a *trans*-translation deficient strain, the lower growth rates are related to slower ribosome recycling. As ribosome availability becomes limiting, the cell redirects translation capacity to producing ribosomal components and away from precursors, *i.e.,* amino acid biosynthesis. This study also allowed us to suggest potential *trans*-translation targets based on the protein levels and peptide distribution

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P44

Proteomics to get multi-organ insights into processes induced during zebrafish fin regeneration

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Objective: Bone fractures are a common injury, and their incidence and severity strongly depend on the bone integrity and health status of the patient. However, bone is a highly regenerative tissue, even though the processes underlying bone regeneration are not fully understood yet. A suitable model system to gain insights into bone regeneration is zebrafish, as they are able to regenerate complex organs, i.e. they can fully recover their fins after amputation. Thereby, amputation causes stress for the whole organism, and it is likely that multiple organs contribute to the final regeneration of the fin. Therefore, we aimed to investigate the effects of zebrafish fin amputation on multiple organs, namely fin, pancreas, liver, brain, and plasma.

Methods: To reveal mechanistic information on the processes triggered by zebrafish fin amputation on multi-organ level, we examined the different organs of male and female zebrafish two and four days after amputation using liquid chromatography- and mass spectrometry-based untargeted proteomics. Notably, amputation triggers both regeneration and wound healing in the first two days. With the formation of a blastema after up to two days, wound healing is complete and only regenerative processes are active. To distinguish regeneration effects from wound healing effects, zebrafish two days after an incision were used as controls. In addition to the induction of regeneration-related processes, gender-specific effects were also investigated. To gain mechanistic insights, significantly altered proteins were determined, pathway enrichment was examined, and a Weighted Gene Correlation Network Analysis (WGCNA) was performed to get further insights into the processes taking place using a p-value-independent network-based approach.

Results: With this study, we identified metabolic pathways and cytoskeleton-related changes in several organs. Furthermore, the analysis revealed gender-dependent differences in the regulation of metabolic pathways, and key proteins relevant for regeneration were identified in the different organs based on WGCNA.

Conclusion: Overall, we demonstrated that fin amputation and subsequent regeneration have effects on whole-body metabolism, with large differences between male and female zebrafish. With these results, we contribute to a detailed mechanistic understanding of regeneration processes that go beyond wound healing alone.

P45

An Integrated Landscape of mRNA and Protein Isoforms

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Cellular processes like alternative splicing (AS) and proteolytic processing generate various proteoforms from the same protein-coding gene. Studies using high-throughput sequencing indicate that about 90% of multi-exon genes in humans undergo AS. These AS events often occur in a tissue and developmental stage specific manner. Proteolytic processing is important for regulation of protein function and involved in processes like cell cycle regulation, apoptosis and protein degradation.

Standard bottom-up proteomics involves digesting proteins into peptides. Since many peptide sequences match to several proteoforms, the information to which proteoform a given peptide belongs is lost at this step. Furthermore, there is an evolutionarily conserved preferential usage of lysine and arginine at splicing junctions. In combination with tryptic digestion, this impedes detection of splice junction-spanning peptides. Hence, the contribution of AS to protein diversity remains understudied.

Here, we combine full-length mRNA sequencing (Iso-Seq) with proteomic analyses to obtain an integrated landscape of mRNA and protein isoforms in human RPE-1 cells. Protein isoforms were resolved by extensive protein-level fractionation by SDS-PAGE using the GELFREE 8100 fractionation system. After digestion of individual fractions and TMT labeling, the samples were combined and analyzed using conventional LC-MS/MS. Abundance profiles of peptides across fractions then provides information about protein isoforms.

We have established a reference set of 40,259 full-length protein-coding transcripts. In addition, we developed a computational framework to automatically detect different proteoforms for 12,400 unique genes. Our data reveals multiple protein isoforms for many genes, for example, 5 out of 5 known isoforms of TMPO. We are currently working on the integration of the proteomic and transcriptomic datasets. Our integrated landscape of mRNA and protein isoforms will provide insights into how transcriptional, translational and post-translational processes contribute to proteome complexity.

P46

Deciphering the WNT/Planar Cell Polarity interactome using proximity labeling

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Introduction

Wnt/ Planar Cell Polarity (PCP) pathway is one of the evolutionary conserved signaling cascades playing inevitable role in the maintenance and establishment of cell polarity. Its deregulation can lead to severe defects such as impaired cellular structures alignment or deregulated migration of cells. Although extensively studied, still few is known about particular molecules involved in the signaling, their interacting partners and relationship with the other Wnt signaling branches.

Objectives

The main objectives were to a) describe the general interactome of the core Wnt/PCP components, membrane receptors ROR1, ROR2 and VANGL2, and cytoplasmic proteins DVL3 and PRICKLE1; and b) find

novel interacting proteins and validate them *in vitro* and *in vivo*.

Material & methods

We applied proximity-labeling biotinylation (BioID) for the investigation of the Wnt/PCP interactome components using stable Tet-ON T-REx-293 cell lines. The samples were measured on Orbitrap Fusion Lumos and the searches were conducted using MaxQuant software. Resulting output was further analyzed using the DEP R package, and external tools as REPRINT, ProHITS, gprofiler2 and Human Cell Map. For *in vivo* validation of selected hits, we used a zebrafish model.

Results

We described the interactome of the core Wnt/PCP components and their respective overlapping interacting partners by two alternative analysis approaches: differential expression by the *limma* test and REPRINT analysis. For the following *in vitro* and *in vivo* biological validation, we applied cluster analysis which showed us 6 possible clusters in the data. We decided to further investigate the cluster comprised of ROR1, ROR2 and PRICKLE1 interactors. We found several interesting novel hits showing a phenotype in the zebrafish model.

Conclusion

We described the general Wnt/PCP interactome using BioID approach and revealed several novel interacting proteins which are now validated *in vitro* and *in vivo*.

Acknowledgements

KG, NZ, MM: Funded by European Structural and Investment Funds, Operational Programme Research, Development and Education – "Preclinical Progression of New Organic Compounds with Targeted Biological Activity" (Preclinprogress) – CZ.02.1.01/0.0/0.0/16_025/0007381.

PP, TR, VB, ZZ: Funded by the Czech Science Foundation EXPRO grant GX19-28347X.

We acknowledge CEITEC Proteomics Core Facility of CIISB, Instruct-CZ Centre, supported by MEYS CR (LM2018127).

P47

Degradation of the marine polysaccharide arabinogalactan by particle-associated bacteria *Maribacter*

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Planktonic microalgae contribute nearly 50% to the global net photosynthesis. In temperate regions a succession of microalgae is usually observed, initially diatoms are dominant followed by *Phaeocystis*. The breakdown of these microalgae fuels heterotrophic bacterioplankton, *Phaeocystis* plays a crucial role in that. Prominent carbohydrates produced by *Phaeocystis* are arabinose and galactose, which are the main components of arabinogalactan (Alderkamp *et al.*, 2007). In a recent study arabinogalactan was confirmed as a main carbohydrate of diatoms (Vidal-Melgosa *et al.*, 2021). The presence of arabinogalactan correlated with the diatom biomass. Currently there is no study available how microalgal arabinogalactan is degraded.

Therefore we studied the proteomes of *Maribacter* strains, a marine particle-associated (PA) genus, grown on polysaccharides to gain insight in the degradation of arabinogalactan and other cell wall polysaccharides. Here we report the proteins expressed by *Maribacter* grown with arabinogalactan as a carbon and energy source.

Maribacter forsetii was grown in liquid medium on several different mono- and polysaccharides, including arabinogalactan and microalgal cell walls. Microalgae were collected during the late spring bloom of 2020 in the German bright of the North Sea. The analysis of the proteins was performed on a liquid chromatography coupled to an orbitrap mass spectrometry. Protein identification and quantification was done using MaxQuant and a strain-specific protein sequence database (Tyanova *et al.*, 2016).

The identified and expressed proteins of microalgal cell wall utilizing cells were similar to the ones of cells grown on arabinogalactan. Both conditions had expressed the same SusC/D outer membrane oligosaccharide transporters. The expressed proteins contained candidate carbohydrate-active enzymes (CAZymes) for the arabinose/galactose pathway. The *Maribacter* strains were able to use either the

glycolysis or the pentose phosphate pathway to boost their citrate cycle. The induced enzymes, which might play a role in the utilization of arabinogalactan, were not located in a polysaccharide utilization locus (PUL), operon like genetic region encoding the proteins for hydrolysis and transport of polysaccharides on the chromosome (Grondin *et al.*, 2017).

Our study showed that *Maribacter* strains do not always use PULs for encoding polysaccharide degrading pathways. It corroborates that arabinogalactan is a major cell wall polysaccharide in microalgae and provided a first insight in the utilization pathway of arabinogalactan by *Maribacter*.

P48

Proteomic profiling of osteogenic and adipogenic bone marrow lineages derived from mesenchymal stromal stem cells by means of quantitative mass spectrometry (SWATH)

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Objective: Mesenchymal stromal stem cells (MSCs) gain increasing focus in the field of regenerative medicine due to their differentiational abilities into chondrocytes, adipocytes and osteoblastic cells. However, it is apparent that the transformation processes are extremely complex, cause cellular heterogeneity and need further insights into functional annotation. The aim of the study was to characterize differences between MSCs and cells after adipogenic or osteoblastic differentiation at the proteome level.

Methods: MSCs were isolated from human bone marrow, cultured and differentiated into adipogenic and osteoblastic cell lineages. Comparative proteomic profiling was performed with electrospray ionization tandem mass spectrometry in data-independent acquisition mode. Proteins were quantified using deep neural network in library free mode along with machine learning algorithms and correlated to the Molecular Signature Database (MSigDB) hallmark gene set collections for functional annotation.

Results: We analyzed 4.108 proteins across all samples which revealed a distinct clustering between MSCs and cell differentiation states. Protein expression profiling identified activation of the *Peroxisome proliferator-activated receptors (PPARs)* signaling pathway after adipocytic differentiation. In addition, two distinct protein marker panels could be defined for osteoblastic and adipocytic cell lineages.

Conclusion: Combination of deep neural network and machine-learning algorithms with data-independent mass spectrometry distinguish MSCs and cell lineages after adipogenic or osteoblastic differentiation. We identified specific proteins as the molecular basis for bone formation which could be used for regenerative medicine, stem cell and cancer research.

P49

A piece of CaKe1 – proteomic study of casein kinase 1 isoforms

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INTRODUCTION

The casein kinase 1 (CK1) family is evolutionarily conserved group of serine/threonine-specific protein kinases consisting of seven isoforms present in human. Those are namely CK1α, α-like, γ1, γ2, γ3, δ and ε. These isoforms share high homology in their kinase domain which is causing major issues especially in the development of isoform specific inhibitors as they are potent therapeutic targets. Therefore, determination of redundancy and uniqueness of events harbored by individual isoforms is crucial for the better understanding of plethora processed in which CK1 isoforms are involved.

OBJECTIVES

Our three main objectives were: a) to identify the effect of depletion of individual CK1 isoforms on the level of whole-proteome and phospho-proteome changes; b) to identify abundant as well as dynamic

protein interactors of individual CK1 isoforms; c) to comprehensively join the three above-mentioned layers of proteomic analysis outputs into the biological map for individual CK1 isoforms.

METHODS

We created T-REx-293 knockout cell lines used for the whole-proteome and phospho-proteome studies using CRISPR/Cas9 technology. Interactome analysis was carried out employing TurboID proximity-labeling biotinylation on the models of stable Tet-ON FlpIn-T-REx-293 cell lines. The samples were measure on Orbitrap Fusion Lumos/Q-Exactive HF-X/Exploris and the searches were conducted using MaxQuant/DIA-NN software. Further analysis was performed using DEP R package and in the case of TurboID were also utilized tools as REPRINT and ProHITS.

RESULTS

We described the effect of depletion of individual CK1 isoforms on the levels of whole-proteome as well as phospho-proteome. We also described the landscape of abundant interactors of individual CK1 isoforms. The most importantly, we were able to capture the change of dynamic interactors upon the activation of canonical Wnt signaling pathway, where majority of CK1 isoforms is involved.

Besides the outcomes and interactors which are already described, that only shows the strength of our analysis, we uncovered also several interesting novel hits elaborating complexity of the CK1 biology.

CONCLUSION

We achieved the comprehensive picture of individual CK1 isoforms which will serve us in the upcoming deciphering of CK1 functional relationships.

ACKNOWLEDGEMENTS

Supported by Czech Science Foundation - Grant projects of excellence in basic research EXPRO - Molecular and functional analysis of casein kinase 1 biology (GX19-28347X).

The main author is Brno Ph.D. Talent Scholarship Holder – Funded by the Brno City Municipality.

We acknowledge CEITEC Proteomics Core Facility of CIISB, Instruct-CZ Centre, supported by MEYS CR (LM2018127).

P50

Large clostridial toxins reorganize proteome and phosphoproteome of target cells

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Large clostridial toxins (LCTs) have been identified as primary virulence factors in various animal and human diseases such as *C. difficile* infection (CDI), wound-associated gas gangrene or toxic shock syndrome. Classification of toxins as LCTs was made basing on a high sequence homology and similar structural set up as well as their ability to induce pronounced changes in cell morphology. The group of LCTs include toxins TcdA and TcdB from *Clostridioides difficile*, TcsL and TcsH from *Paeniclostridium sordellii*, TcnA from *Clostridium novyi* and *Clostridium perfringens"* TpeL.

Proteome and phosphoproteome responses of *C. difficile* toxins and *C. novyi"s* TcnA were analyzed in cell culture to elucidate unknown functions of these pathogens and their toxins. A combinational approach of biochemical analysis and different MS techniques such as shot-gun proteomics and MRM were used.

Comprehensive SILAC, TMT-labeled and label-free (phospho-) proteome analysis had been performed to investigate effects of TcdA, TcdB, CDT and TcnA on target cells in vitro. Cell-cell junction, cytoskeleton organization and cell death were found to be toxin-responsive. Toxic effects of TcdA could be traced back to its glucosyltransferase activity while in case of TcdB a catalytically inactive variant also altered the proteome of target cells and induced early cell death. By infection of mice with *C. difficile* spores various biological processes related to immune response were identified as enriched while metabolic processes were found to be down-regulated. Administration of TcnA lead to downregulation of DNA damage response on proteome level, while the same pathway was significantly up-regulated on phosphoproteome analysis. A similar behavior could be observed for Rho

Family GTPase signaling, which was enhanced on proteome level but strongly down-regulated on phosphoproteome level. All applied toxins had an impact on the actin cytoskeleton of target cells.

Utilizing various MS techniques it was possible to illuminate so far unknown effects of *C. difficile* and its toxins as well as *C. novyi"s* TcnA on cellular processes of the host.

P51

Acclimatization of *Methylocystis* sp. strain SC2 to high NH_4^+ load by multiple proteomic and metabolic response mechanisms

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Proteobacterial methane-oxidizing bacteria (MOB) are able to utilize methane (CH_4) as their sole source of carbon and energy. Their key enzyme is particulate methane monooxygenase (pMMO) that oxidizes CH_4 to methanol. Ammonium (NH_4^+) acts not only as an important N source for methanotroph growth but can also have an inhibitory effect on CH_4 oxidation and growth, in particular at high NH_4^+ load. The competition between NH_3 and CH_4 for the active site of the pMMO leads to the formation of toxic hydroxylamine. In particular, the intracellular acclimatization of alphaproteobacterial MOB to increasing NH_4^+ load is not yet well studied. Here, we applied a combination of physiological growth experiments, global proteomics, profiling of intracellular amino acids, and measurement of NOx compounds to elucidate the acclimatization response of the alphaproteobacterial *Methylocystis* strain SC2 to increasing NH_4^+ concentrations.

Under CH_4 replete conditions, NH_4^+ concentrations higher than 30 mM triggered an extended lag phase and strongly diminished the growth rate of strain SC2. However, regardless of the ammonium concentration (1 to 75 mM NH_4^+), SC2 growth always yielded the nearly same biomass after prolonged incubation. The addition of 100 mM NH_4^+ had a complete inhibition effect on SC2 growth. Increasing NH_4^+ load induced multiple acclimatization mechanisms. The ionic stress effect of high NH_4^+ loads triggered the activity of the "salt-in" strategy of potassium (K^+) accumulation, production of various stress-responsive genes, and synthesis of proline as an osmoprotectant. A total of 438 proteins were differentially regulated (DRPs), with a significant relationship between the number of DRPs and the increase in NH_4^+ load. The change in apparent *Km* value for methane oxidation to 2.72 µM (30 mM NH_4^+) and 3.40 µM (50 mM NH_4^+) relative to 0.34 µM under standard growth condition (1 mM NH_4^+) mirrors the increased inhibition effect of excess NH_4^+ on CH_4 oxidation. The latter triggered a significant upregulation of the hydroxylamine detoxification pathways, thereby resulting in a greatly increased production and cellular release of nitrite (NO_2^-) and nitrous oxide (N_2O).

In conclusion, the acclimation limit of strain SC2 to 75 mM NH_4^+ is presumably due to cellular need to combat both osmotic stress and the increasing production of toxic hydroxylamine.

P52

Two-step enrichment facilitates background reduction in proteomic analyses of lysosomes

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Objective

Lysosomes are cytoplasmic organelles found in almost all mammalian cells. While they have long been known for their degradative functions, they are recently emerging as regulators of cellular metabolism and shown to be involved in various diseases. Therefore, there is an increasing interest in the lysosomal proteome. To allow its unbiased investigation via mass spectrometry (MS), lysosome enrichment is a prerequisite. We compared (and combined) two of the most efficient approaches for lysosome enrichment. Superparamagnetic iron oxide nanoparticles (SPIONs) and the immunoprecipitation from cells overexpressing TMEM192-3xHA (TMEM-IP). We showed that both approaches enrich complementary fractions of the lysosomal proteome and that their sequential combination allows to reduce the number of proteins originating from contaminating organelles.

Methods

HEK 293 WT and TMEM192-3xHA overexpressing cells were used in four biological replicates. Lysosomes were enriched using SPIONs and TMEM-IP in different combinations, enrichment was validated by enzymatic

assays and western blot. Enriched fractions were digested using single-pot, solid-phase-enhanced sample preparation (sp3) with LysC and trypsin. Peptides were desalted and analysed using an Orbitrap Fusion Lumos by data-dependent and -independent acquisition (DDA/DIA). Data analysis and label-free quantification was performed with Spectronaut.

Results

To investigate the performance of individual enrichment protocols and to assess their effect on the lysosomal proteome, different conditions and combination of thereof were applied: TMEM-IP, SPIONs and SPIONs followed by TMEM-IP. Furthermore, SPIONs enrichment was performed from cells overexpressing TMEM192-3xHA and TMEM-IP from such treated with SPIONs. This allowed to compare efficiencies of individual approaches and to assess if respective experimental setup affected lysosomal proteome. A total of 6,172 protein groups were identified, of which 469 were known as lysosomal. SPIONs and TMEM-IP performed largely similar with differences in the abundance of individual proteins. From flow through fractions of SPIONs enrichment column, intact lysosome could be recovered by TMEM-IP, representing lysosome not involved in the endocytic pathway. When sequential SPIONs and TMEM-IP was performed, lower lysosomal recovery rates were observed, but purity of lysosomal fraction increased. Based on unsupervised clustering and principal component analysis, the two TMEM-IP and the SPIONs samples clustered, while the two-step approach showed a different behaviour.

Conclusion

Lysosome enrichment by SPIONs and TMEM-IP are both effective. Each approach results in a different set of proteins with a high overlap in those known to be of lysosomal localization and quantitative differences of the latter. Combination of both approaches allows to reduce unspecific enrichment of background proteins.

P53

A Proteomics Approach for Profiling Redox-Sensitive Changes in Mesothelial Cells during Experimental Peritoneal Dialysis

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Peritoneal dialysis (PD) is a life-saving renal replacement therapy, which uses the peritoneum as semi-permeable membrane to remove uremic toxins and water from the patient by a glucose-based PD fluid (PDF). The special composition of these hyperosmotic PDFs triggers morphological and functional changes in the peritoneal membrane. The high glucose concentration, low pH, non-physiological buffer and glucose degradation products contained in the fluids lead to generation of reactive oxygen species (ROS) in the peritoneal cavity, leading to impaired cell viability and progression of PD-related vasculopathy, hyper-vascularization, and diabetes-like damage of vessels, eventually leading to failure of the technique. Here, we aim to analyse the specific targets of ROS during PD and the impact and of a potentially anti-oxidative pharmacological intervention with glutamine-supplementation of the PDF.

To establish a redox-proteomics workflow for studying the targets and mechanisms of oxidative stress in peritoneal mesothelial cells we used a gold-standard model of redox-stress (H2O2) and PDF-induced stress. Elevated levels of oxidative stress were first validated by increased abundance of intracellular ROS and increased superoxide dismutase activity with PDF and H2O2 treatment; a reduction of these parameters was observed with added glutamine. To detect alterations of the redox proteome, cysteine residues were either directly or indirectly labelled with isobaric tags (iodo-TMT). The LC/MS-based redox proteomics workflow was optimized regarding cell lysis and labelling buffer conditions to yield improved labelling efficiency, as well as blocking agents and concentrations, affinity tag-enrichment of labelled peptides, elution conditions and reproducibility. The optimized protocol enabled identification of ~7600 proteins and ~2400 proteins affected by redox-stress. We found that mesothelial cells exposed to H2O2, PD fluid or PD fluid supplemented glutamine show specific differences in their oxidation status.

With the established workflow, we were able to detect a higher oxidation status of PD treated cells compared to control cells and a lower oxidation status of the

samples supplemented with glutamine compared to PD fluid without. Redox proteomics of peritoneal cells may represent a novel approach for the identification of PD-pathomechanisms and evaluation of clinically relevant anti-oxidative interventions.

P54

Peptide concentration affects RapiGest precipitation

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Question

RapiGest is an acid-cleavable MS-compatible surfactant which is structurally related to SDS. It facilitates the unfolding of proteins, and enhances the proteolytic digestion of proteins in-solution, especially for highly hydrophobic domains. After proteolytic digestion, RapiGest can easily be removed from samples by acid hydrolysis and precipitation, allowing for a straightforward sample clean-up. We observed a high variability in RapiGest precipitation between samples of different origin, resulting in an incomplete removal of its hydrophobic moiety, therefore potentially impeding subsequent MS-sample preparation and analysis. In this study, we investigated the sample properties which are causative for these precipitation inconsistencies and developed a strategy to reliably remove RapiGest.

Methods

The influence of salt/acid concentration, sample viscosity/density, and peptide concentration on RapiGest precipitation was tested using tryptic digests of bovine serum albumin (BSA). RapiGest hydrolysis was investigated using thin layer chromatography. Residual trigger proteins were removed with C8 reversed phase StageTips. Potential peptide losses related to RapiGest precipitation and sample exposure to C8 material were investigated using tryptic digests of BSA, HeLa whole cell lysate, and a six-protein digest consisting of BSA, β-lactoglobulin, ovalbumin, carbonic anhydrase, α-casein, and β-casein. LC-MS/MS analyses were conducted on an UltiMate 3000 nanoUHPLC coupled to an Orbitrap Fusion Lumos mass spectrometer. Raw data were searched with MaxQuant and follow-up analyses performed with Perseus, MS Excel, and Graphpad Prism.

Results

Of the various sample parameters tested, we identified peptide concentration to be the main factor influencing RapiGest precipitation. Increasing peptide concentration positively correlated with RapiGest pellet size. As these results implied that peptides co-precipitate with RapiGest, we evaluated potential peptide losses using a six-protein digest, for which we did not observe tremendous differences to control samples. To facilitate the removal of RapiGest from low-concentration samples, we investigated if addition of intact trigger proteins can induce RapiGest precipitation. We were able to show that addition of proteins does not only result in RapiGest precipitation, but that also the majority of intact proteins is co-precipitated. To remove residual trigger protein, we devised a strategy using C8 reversed phase material. While trigger protein addition as such did not result in samples losses, subsequent removal of residual trigger proteins by C8 material resulted in reduced signal intensity in a subset of peptides from HeLa whole cell lysate tryptic digests.

Conclusion

We show that RapiGest precipitation depends on peptide concentration, that addition of intact proteins can trigger it in low concentrated samples, and that residual trigger proteins can be removed by C8 material.

P55

Investigation of crucial sample preparation steps in the proteomics workflow

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Sample preparation is a crucial step for quantitative proteomics analysis to ensure repeatability and robustness of the results. There is no universal sample

preparation method and every single sample would certainly benefit from a dedicated protocol optimisation. However, unavoidable steps and recurrent questions remain, especially when dealing with non serum-free cell cultures and/or with samples presenting low amounts of proteins. In this context, we evaluated the extent of remaining bovine serum protein contaminations in human cell cultures as well as the impact of different protein extraction buffers. In parallel, the effect of sample amount reduction down to 1000 cells using miniaturized acrylamide photopolymerized tube gels was assessed. It is a challenge to lose as less as possible material during sample preparation. Investigations are in progress using either focused-ultrasonication from Covaris or sample homogenenization with PreOmics BeatBox to further improve proteins extraction.

A bovine serum concentration range was applied on B lymphocytes cell cultures. Cells were washed 5 times with cold PBS before pelleting. RIPA and Laemmli buffer were used to evaluate yield, efficiency and repeatability of protein extraction. In parallel, total proteins were extracted from mouse macrophages using urea-based buffers. Miniaturized photopolymerized tube gels were prepared to deal with the low sample amount, namely 10000 and 1000 sorted cells. Preomics BeatBox and Covaris focused-ultrasonication technologies were tested on several sample types including PBMC cells, skin and saliva prior to protein digestion. LC-MS/MS analyses were performed on an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific) and on a nanoAcquity Q Exactive Plus coupling (Waters, Thermo Scientific). Data were interpreted using Proline,Maxquant and Prostar software.

Overall, identification and quantification results demonstrate that with five efficient PBS washes, serum proteins can be properly removed and remain very low abundant in the samples. Thus, no major difference was observed when working with high or low amounts of bovine serum and our results confirm the proteomics workflow compatibility with serum-fed cell cultures provided efficient washing steps are undergone. RIPA and Laemmli buffers showed similar results regarding the number of validated proteins/peptides as well as the localization of identified proteins in B lymphocytes cells. Promising results were obtained when working with low sample amounts with more than 1400 and almost 700 proteins quantified from respectively 10000 and 1000 sorted murine macrophage cells using miniaturized acrylamide photopolymerized tube gels.

We investigated several key aspects in the proteomics sample preparation workflow. These results are promising in the context of aiming to work on ever-reduced amounts of starting material, an absolute prerequisite for working with limited cell numbers and ultimately single cells.

P56

Smart Detection Methods for Protein: Protein Interactions in Living Cells

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The interaction of proteins is fundamental to almost all cellular processes. Aberrations within this interaction network are linked to a multitude of pathological conditions. Together, this renders protein:protein interaction (PPI) an important target from basic research to drug development. Here we introduce the bioluminescent NanoBiT® and NanoBRET™ technology platforms that enable to study protein interactions in a live-cell context.

NanoLuc® Binary Technology (NanoBiT®) is a structural complementation reporter based on NanoLuc® luciferase (Fig. 1, right). It is composed of two engineered subunits – LgBiT (*Large BiT*; 18 kDa) and SmBiT (*Small BiT*; 1.3 kDa) that exhibit minimal self-association. The interaction of two proteins that were fused to LgBiT and SmBiT respectively facilitates the reconstitution of the functional NanoBiT® luciferase. The small size of the fusion tags minimizes interference with normal protein function while their low intrinsic affinity mitigates unspecific signal and enables real-time monitoring of interaction dynamics.

NanoBRET™ represents a powerful alternative to interrogate homo- and heteromeric protein interactions (Fig. 1, left). In this proximity-based assay, NanoLuc® is used as a BRET energy donor and fluorescently labeled HaloTag® as the energy acceptor. When fused to proteins of interest, this technology enables sensitive, reproducible detection of protein interactions within their cellular context.

This presentation will highlight the respective advantages of NanoBiT® and NanoBRET™ to analyze PPI dynamics and discuss the key steps of their workflows.

Poster presentation

Fig. 1

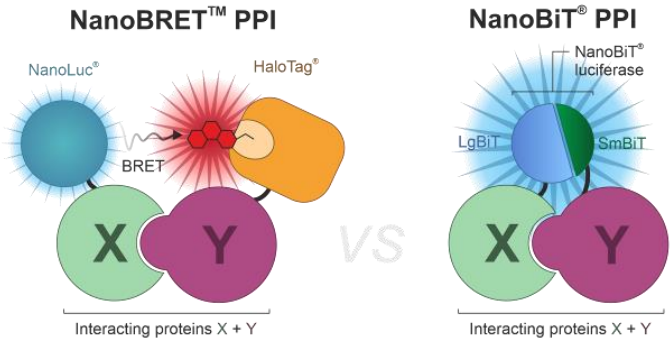
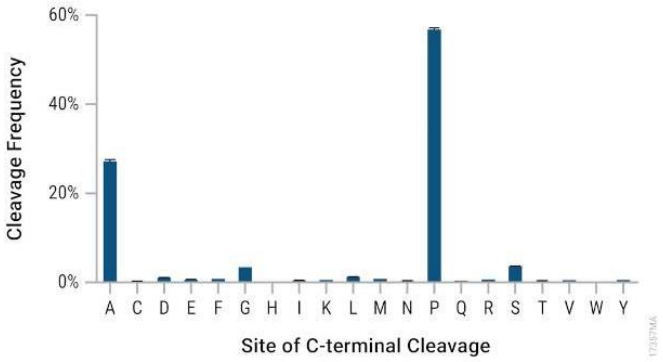


Fig. 1



P57

Characterization of Therapeutic Antibodies with a Protease that Cleaves after Proline & Alanine

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Proteases beyond trypsin are important for protein characterization by mass spectrometry since they help increase sequence coverage and identify posttranslational modifications. Like trypsin, the most commonly-used alternative proteases also cleave at charged residues thus there is a need for proteases that cleave at unique sites in the proteome. Here we describe the characterization of ProAlanase, a protease that preferentially cleaves proteins on the C-terminal side of proline and alanine residues (Figure 1). Digestion with ProAlanase is optimal with short durations of 1-2 hours at acidic pH (~1.5-2.0) which suppresses introduction of sample preparation artifacts and minimizes nonspecific digestion. The enzyme digested IgG successfully under both reducing and non-reducing conditions which facilitated characterization of primary structure coverage, PTMs, and disulfide bonds.

P58

Highly Specific and Autoproteolytically Resistant Trypsin for Accurate Protein Mass Spec Analysis

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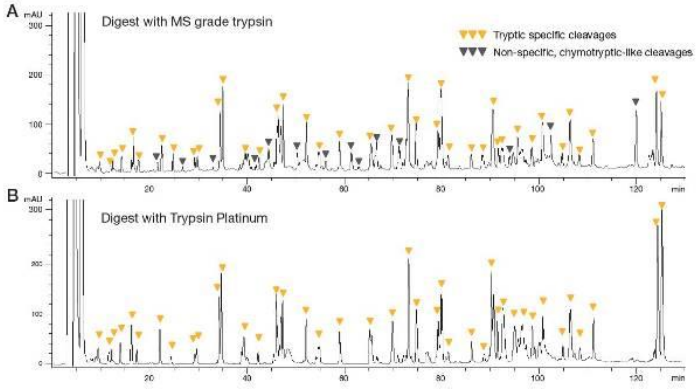
Trypsin is the most popular protease in protein mass spec field owing to robust performance, optimal distribution of tryptic cleavage sites in proteins and strong charge of trypsin generated peptides. To meet requirements of protein mass spec analysis, trypsin must demonstrate high cleavage specificity and stability. In this study, we investigated trypsin properties in detail. The commonly used commercially available Proteomics and MS grade trypsins showed low, but detectable non-specific cleavage specificity. They also demonstrated prominent autoproteolysis. Non-specific and tryptic autoproteolytic peptides became abundant if large trypsin quantities were used. This compromised protein mass spec analysis. To address the problem, we developed new trypsin that was free of non-specific activity (Figure 1), had high autoproteolytic resistance and increased proteolytic efficiency.

Poster presentation

Fig. 1



P59

***Ex vivo* characterization of the transcription factor IRF4 interactome in T helper cell subsets**

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Objective

IRF4, a key regulator in lymphoid- and myeloid-cell differentiation, promotes the maturation of T helper (Th) cell populations into effector cells. Despite its central role in Th lineage determination, molecular mechanisms of IRF4-mediated gene expression *in vivo* are still poorly understood and the question of how IRF4 interacting proteins steer IRF4-mediated target gene transcription in different Th cell subtypes remains unresolved to date. Here, we present an optimized proteomic workflow enabling the *ex vivo* characterization of transcription factor interactomes combining chemical cross-linking and affinity purification of biotinylated target protein complexes.

Methods

To generate *in vivo* biotin-tagged IRF4, we crossed transgenic mice that express IRF4 coupled to a BirA recognition site with the ROSA26^{BirA} strain [1]. The offspring (IRF4^{Bio} mouse) expresses biotinylated IRF4 at endogenous levels. Naïve CD4+ T cells were isolated from splenic single-cell suspensions by negative selecting magnetic-activated cell sorting (MACS). After *ex vivo* differentiation, Th cell subsets were treated with a chemical crosslinker, harvested, and lysed. Biotinylated IRF4 along with its interactors was captured using magnetic streptavidin beads. After tryptic digestion, peptides were analyzed by LC-MS on an Orbitrap Exploris 480 instrument in DIA mode.

Results

In the present study, we focused on the characterization of the transcription factor IRF4 and its interplayers in *ex vivo* propagated CD4+ T cell subsets, for which we developed a robust and highly reproducible affinity purification protocol: Combing reversible chemical cross-linking, optimized wash and elution steps as well as SP3 (single-pot solid-phase-enhanced sample preparation) [2] for MS sample processing, we were able to markedly reduce unspecific background as well as contamination derived from the streptavidin beads during pulldown experiments while concomitantly preserving weak and transient interactions.

We could describe a "core IRF4 interactome" which is preserved even in functionally opposed T cell subsets like Treg and Th17 cells. In addition, we also detected lineage-specific interactors involved in IRF4-steered gene expression including novel, yet uncharted interplayers.

Conclusion

We developed an optimized affinity purification protocol for the *ex vivo* characterization of transcription factor interactomes in (differentiating) immune cells.

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P60

The role of neprilysin in degrading the mitochondrial proteome

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Objective The main aim of the present work was, initially, to describe the aberrant mitochondrial proteolytic activity in a model of altered dopamine homeostasis. The first results prompted us to further investigate the complete set of proteins degraded by neprilysin.

Methods We developed and applied the mitochondrial dimethylation-TAILS (Terminal Amine Isotopic Labeling of Substrates) degradomics approach, to identify mitochondrial proteins which were proteolytically processed in a cellular model of Parkinson"s Disease (PD) (*i.e.*, altered dopamine homeostasis in neuroblastoma SH-SY5Y cells). By the analysis of cleavage sites, we identified an aberrantly activated protease in our model [1]. Afterwards, we applied a mitochondrial sub-fractionation protocol (*i.e.*, mitochondrial enrichment based on the use of surfactants coupled to sodium carbonate treatment). Lastly, we applied a classical dimethylation-TAILS approach for the identification of the complete set of substrates of our candidate protease.

Results We identified neprilysin, a zinc-dependent endopeptidase ubiquitously distributed, as a candidate protease aberrantly activated in our PD model. We also demonstrated for the first time that the human neprilysin peptidase was enriched in the mitochondrial subcellular fraction [1] and it is present as a soluble protein within mitochondria, likely residing in the matrix of the organelle. Moreover, we showed a significant co-localization between neprilysin and mitochondria by immunofluorescence, even though neprilysin was also diffusely distributed in the cytoplasm. Eventually, by the application of the standard dimethylation-TAILS workflow, we discovered novel protein targets of neprilysin.

Conclusion We collected compelling evidence of an aberrant function of mitochondrial proteases upon dopamine homeostasis disruption and we identified for the first time neprilysin as a candidate mitochondrial protease hyperactivated in this condition. The protein targets degraded by neprilysin can represent crucial factors in mitochondrial dysfunction linked to dopamine imbalance in PD.

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P61

Proteomic analysis of the interactome of the autophagy receptor TAX1BP1 (T6BP) in model antigen presenting cells

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CD4+ T lymphocytes play a major role in the establishment and maintenance of immunity. They are activated by antigenic peptides derived from extracellular or newly synthesized (endogenous) proteins presented on the surface of antigen presenting cells (APCs) by the MHC-II molecules. The pathways leading to endogenous MHC-II presentation remain poorly characterized. We demonstrate here that the autophagy receptor, T6BP, influences both autophagy-dependent and -independent endogenous presentation of HIV- and HCMV-derived peptides. To determine the interactome of T6BP, model APCs were transfected with a plasmid encoding GFP-T6BP, a construct that was previously functionally characterized (Morriswood et *al.*, 2007), and as negative control, a plasmid encoding GFP. We then performed a large-scale immunoprecipitation (IP) using GFP as target. The immunoprecipitation products (three biological replicates per condition) were trypsin-digested and analysed with a nanoElute-timsTOF Pro LC-MS/MS system (Bruker). We identified 116 high-confidence T6BP proximal proteins included previously known T6BP-interactants and novel potential partners

like calnexin. Remarkably, calnexin silencing replicates the functional consequences of T6BP silencing: decreased CD4+ T cell activation and exacerbated CD74 degradation. Altogether, we unravel T6BP as a key player of the MHC-II-restricted endogenous presentation pathway.

The Autophagy Receptor TAX1BP1 (T6BP) is a novel player in antigen presentation by MHC-II molecules. Mathias Pereira, Clémence Richetta, Gabriela Sarango, Anita Kumari, Michael Ghosh, Lisa Bertrand, Cédric Pionneau, Morgane Le Gall, Sylvie Grégoire, Raphaël Jeger-Madiot, Elina Rosoy, Mathias Faure, Audrey Esclatine, Stéphanie Graff-Dubois, Stefan Stefanović, Bénédicte Manoury, Bertha Cecilia Ramirez, Arnaud Moris. bioRxiv 2021.04.21.440798; doi: https://doi.org/10.1101/2021.04.21.440798

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P62

Dynamic Intracellular Metabolic Cell Signaling Profiles During Antigen-Dependent B-Cell Differentiation

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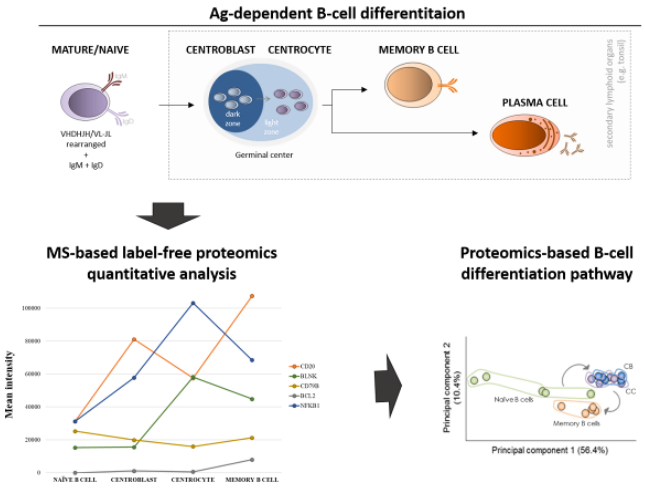
Human B-cell differentiation has been extensively investigated on genomic and transcriptomic grounds; however, no studies have accomplished so far detailed analysis of antigen-dependent maturation-associated human B-cell populations from a proteomic perspective. Here, we investigate for the first time the quantitative proteomic profiles of B-cells undergoing antigen-dependent maturation using a label-free LC-MS/MS approach applied on 5 purified B-cell subpopulations (naive, centroblasts, centrocytes, memory and plasma B-cells) from reactive tonsils. Our results revealed that the actual differences among these B-cell subpopulations are a combination of expression of a few maturation stage-specific proteins within each B-cell subset and maturation-associated changes in relative protein expression levels which are related with metabolic regulation. The considerable overlap of the proteome of the 5 studied B-cell subsets strengthens the key role of the regulation of the stoichiometry of molecules associated with metabolic regulation and programming, among other signaling cascades (such as antigen recognition and presentation and cell survival) crucial for the transition between each B-cell maturation stage.

Fig. 1



P63

Exploring dynamic proteome and metabolome of HepG2 cell line: rise and fall

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Question

Most of the fateful decisions in the cell's life are performed at the proteome and metabolome levels. However, the methods used to characterize the status of cells and search for potential marker molecules mainly focus on analyzing a particular "stationary" state. Such an analysis is principally limited by the genomic directive. It does not reflect changes that develop in response to external influences or the cell cycle stage changes.

Methods

In this work, we analyzed the dynamics of coordinated changes in the proteome and metabolome of the HepG2 cell line at different stages of its cell cycle. We used panoramic methods of the proteome (LC-MS/MS, Dionex Ultimate 300/Thermo Fisher Orbitrap Fusion) and metabolome (GC×GC/MS, LECO Pegasus 4D BT)

profiling of HepG2 cells, thus analyzing the proteins and metabolites content at five time points. In addition to investigating changes in the proteome and metabolome of the "standard" cell line, we also analyzed the HepG2 profiles with the knocked out TOMM34 gene associated with the development of oncological processes.

Results

We paid particular attention to the "molecular" features traditionally attributed to cancer cells. We monitored changes in the content reflecting the synthesis of amino acids and nucleotides, glycolysis, oxidative phosphorylation, apoptosis, etc. The study made it possible to assess the variability of the proteome and metabolome of the cell line, which is often used as a preclinical model of hepatoblastoma, revealing the influence of technical and biological replicates on the reproducibility.

Conclusions

We believe that the obtained data will increase the experimental transparency of multi-ohm tests, thereby strengthening the reliability of fundamental studies of molecular mechanisms implemented in HepG2 cells and practical work on creating diagnostic and drug tools that could be used in the treatment of hepatoblastoma.

This work was supported by the Russian Science Foundation (RSF Grant #20-14-00328; http://www.rscf.ru/).

P64

Absolute quantification of glycosyltransferases in the Endoplasmatic Reticulum of human cells

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More than half of all human proteins are glycosylated. Among the three major pathways for protein glycosylation in the Endoplasmatic Reticulum, N-glycosylation, O-mannosylation and C-mannosylation, the production of the dolichol linked N-glycan precursor is the most complex pathway consisting among other proteins of more than ten glycosyltransferases, asparagine linked glycosylation (ALG) enzymes. Mutations within this pathway often lead to severe congenital disorders of glycosylation (CDG) in human. Even so, knowledge about this pathway is mostly based on yeast genetics, because the ALG proteins are low abundant transmembrane proteins.

To shed more light on the molecular mechanism of this important pathway, we use shotgun proteomics and multiple reaction monitoring to quantify the members of this pathway in HEK 293T cells and fibroblasts of CDG patients. To determine the abundance-based ratios between the different members of this pathway there are several methods for absolute quantification such as protein standard based absolute quantification (PSAQ) (1) or intensity based absolute quantification (iBAQ) (2). PSAQ is the gold standard for absolute protein quantification, but difficult to achieve. As an alternative we test stable isotope-labeled protein standards for absolute quantification using mass spectrometry (3). Intensity based absolute quantification (iBAQ) has been successfully used to determine the stoichiometry of large protein complexes (4), but as a statistical method it heavily relies on the number of peptides detected for a protein. To overcome the problems associated with low abundant proteins, we used membranes enriched by ultracentrifugation in a low salt sucrose and a high salt sucrose cushion. The target proteins were further enriched by fractionation using SDS PAGE.

We show that most of the ALG enzymes are present in quite similar amounts and it is tempting to speculate that they are organized in protein complexes. These results are discussed, but also pitfalls and shortcomings of the methods used.

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P66

Global Protein Degradation Kinetics Highlight the Role of Mitochondrial Inner-Membrane Proteases in Maintaining Stoichiometry of Respiratory Chain Complexes

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Many mitochondrial protein complexes have a dual genetic origin, with most members encoded by the nuclear genome and some by the mitochondrial genome. Synthesis and degradation rates of proteins originating from both genomes need to be coordinated to ensure complex stoichiometry. The global analysis of protein degradation kinetics allows us to monitor the stability of subunits from different mitochondrial respiratory chain complexes and other proteins in order to enhance our understanding of mitochondrial protein homeostasis.

We have previously combined metabolic pulse-chase labeling with mass spectrometry to quantify protein degradation kinetics on a proteome-wide scale. These data revealed that some proteins are unstable in the first few hours post translation and become more stable afterwards. This non-exponential degradation (NED) affects many subunits of multi-protein complexes, suggesting that complex formation stabilises proteins while excess subunits are degraded by the proteasome.

Here, show that many subunits of mitochondrial respiratory chain complexes that are encoded by the nuclear genome are non-exponentially degraded. Inhibition of mitochondrial translation increases their degradation, suggesting that mitochondria-encoded subunits are rate-limiting factors for complex assembly. NED of mitochondrial proteins is largely independent of the proteasome. In contrast, knocking-out the mitochondrial inner membrane AAA-proteases AFG3L2 (m-AAA) and YME1L1 (i-AAA) stabilised many mitochondrial NED proteins. We conclude that these AAA-proteases mediate NED of excess nuclear-encoded mitochondrial proteins. In summary, our data highlight the importance of mitochondrial proteases to coordinate the formation of protein complexes encoded by mitochondrial and nuclear genomes.

P67

Characterization of ALMS1 protein-protein interaction and function

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Objective

Alström Syndrome is a rare autosomal-recessive disorder, that occurs in 1 to 9 out of 1 million individuals. More than 300 mutations in the ALMS1 gene are known causing a broad range of clinical defects most notably retinal degeneration, Type 2 Diabetes, and truncal obesity. The ALMS1 gene codes for a ~0.5 MDa protein that localizes at the centrioles and the basal body of cilia. Cilia are microtubule-based cell protrusions, which are involved in multiple cellular and signalling processes. The basal body is required for the ciliary structure itself and is involved in polarized cilia docking and selective transport to and into the cilium via the transition zone. So far, the molecular function of ALMS1 and how its mutations cause or influence the severity of the Alström disease in ciliated organs remain unknown. ALMS1 protein is thought to be involved in cilia maintenance, transport and signalling processes. However, also due to its protein size which makes ALMS1 analysis difficult, the functional relevant protein complexes of ALMS1 are not described and its cellular role is not well understood. Here, we aimed at understanding the role of ALMS1 via description of protein-protein interaction on the one hand, and analysis of its (mal-) function in different cilia-related processes in wildtype as well as in mutant cells on the other hand.

Methods

First, we wanted to gain more information about the ALMS1 protein complex by analysis of endogenously tagged ALMS1 in Hek293T cells generated using the CRISPR/Cas9 technique. Using endogenously tagged cells enabled the investigation of ALMS1 protein-protein interaction in ciliated cell culture. Second, ALMS1 bait purification followed by the application of pig retina lysate was used to investigate photoreceptor-specific protein interactions. To understand the molecular function of ALMS1, mutant retinal pigmented epithelial cells were generated using CRISPR/Cas9. Cilia, cilia-dependent signalling and,

based on the interaction data, complex protein localization was investigated.

Results

Using endogenously tagged ALMS1 Hek293T cells allowed purification of ALMS1 protein and its interactors, which are involved in cilia basal body structure and function. In addition, signalling components were identified which are in involved in cilia-dependent and cell cycle processes. Interestingly, ALMS1 mutant cells showed normal cilia regarding length and number. However, a decreased proliferation rate and instable basal body protein composition could be described, which might lead to instability and/or protein transport defects.

Conclusion

Endogenously protein tagging using CRISPR/Cas9 helped to circumvent problems using ectopic expression of full length ALMS1. The cells could be used to investigate protein complexes in ciliated cells and enable further analysis of dynamic (signalling-) dependent processes. These findings might help to understand the still uncertain ALMS1-related role in disease severity.

P68

Neuroglobin modulates the proteome of human neuroblastoma cells inducing the energy metabolism and the autophagic flux

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Neuroglobin (NGB) is an oxygen-binding protein that exerts a crucial neuroprotective function in neuronal cells. The biological effects of NGB strictly depend on its expression levels; in fact, in conditions of stress and hypoxia or when molecules such as 17β-estradiol are administered, the levels of NGB are positively induced [1,2,3]. However, the mechanisms driven by overexpressed NGB are not clear.

In this work, NGB levels were stably induced in a cellular model of human neuroblastoma SH-SY5Y cells by promoting the genetic expression of a NGB-FLAG construct into the genome. In parallel, a wild type neuroblastoma cell line was transfected with an empty vector and used as control cell line (CTRL). A shotgun LFQ proteomics approach was then used to compare those cell lines, with the aim of identifying the major processes triggered by NGB overexpression [4,5].

Proteome analysis revealed that 178 proteins were differentially regulated in NGB-FLAG samples versus CTRL, whereas 107 were up-regulated and 71 down-regulated. Globally, bioinformatics and cluster analyses revealed the enrichment of mitochondrial transport and bioenergetic pathways as biological processes, and some proteins related to lysosome function were highlighted.

Functional validation of proteomics data revealed an increase in the ATP production and mitochondrial mass, in combination with an increased autophagic flux. Thus, we identified a hub that connects the overexpression of NGB and its protective function in neuronal cells [6].

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P69

Brain Proteomic Signatures of an *in vivo* Model of Multiple Sclerosis

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Proteomics, the study of proteins expression, is widely used to investigate molecular pathways and to find biomarkers for complex and heterogeneous diseases such as multiple sclerosis. As a matter of fact, proteins have several important biological functions and play a crucial role as signaling pathways mediators. In this context, we used a high pH reversed-phase fractionation approach coupled to Tandem Mass Tag (TMT)-based proteomics to quantify intact and phosphorylated proteins. We performed such approach on the corpus callosum region of extracted brains from a focal experimental autoimmune encephalomyelitis (EAE) *in vivo* model. Five conditions were chosen namely non-immunized, immunized, immunized with a focal injection of cytokines, immunized with a focal injection of phosphate-buffered saline and treated with minocycline. All experiments were conducted in triplicates. Data allowed to shed light on proteins altered in the different conditions and finding which molecular pathways are involved through a functional analysis using Ingenuity Pathway Analysis.

P70

Environmental Proteomics in Wastewater-Based Epidemiology (EP-WBE). Determination of large biomolecules as biomarkers of population health and activities

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Objective

Many peptides and proteins constitute important biomarkers in clinical practice. Recently, we demonstrated, using proteomic approaches, that wastewater sampled at a wastewater treatment plant (WWTP) contains a huge range of proteins from different species, including human proteins known as disease biomarkers (1). Information carried by peptides and proteins in wastewater can be thus of high value for WBE monitoring but also for monitoring environmental status and human activity (2). Despite the importance of these biomolecules, the protein composition of wastewater was practically unknown until now.On this basis, we have studied the differences of wastewater protein composition in 10 WWTP in Catalonia which serve communities of different population and industrial activity.

Methods

Up to 100 mL of 24-h composite wastewater samples were processed. Samples were filtered and the soluble part was concentrated in a SDS-PAGE gel. Gel slides were digested with trypsin and the resulting peptides analyzed by MALDI-TOF-MS (4800 TOF/TOF) and by HR-LC-MS/MS (Orbitrap-Velos coupled to an Agilent nanoLC). Database search was done using the complete Uniprot database thought the Proteome Discoverer software and a semiquantitative analysis was performed based on the area of the unique detected peptides.

Results

We have been able to identify hundreds of proteins of human origin and other species. Some of the identified

human proteins such as uromodulin, α-amylase, and S100A8 are well-known molecules proposed as biomarkers of health associated with kidney function or stress. The analysis of the protein profile of other species (rat, mouse, cattle and pigs, poultry) has allowed us to find markers on industrial activity in the different areas studied (Fig. 1).

Conclusion

Environmental Proteomics (EP) applied to wastewater provides of a new set of potential human biomarkers for WBE. It allows also monitoring of characteristic peptides from other species such as rat, mouse, cattle, chicken as well as of a large number of bacteria. Thus, wastewater carries a huge variety of proteins that constitute a rich source of biological information. These proteins can be a good indicator of the health status of the population and the diseases prevalent in an area. They also reflect the types of human activity (industry, agriculture and livestock).

The molecules of human origin present in wastewater come mainly from feces and urine and are transported in the sewage system along with many other compounds. Thus, wastewater is part of a complex universe of biochemical signals that represent the population served by a specific water collection system. The analysis by PCR of SARS-CoV-2 in wastewater for the monitorization of the COVID-19 epidemic status in a population is today a paradigmatic example of the WBE approach.

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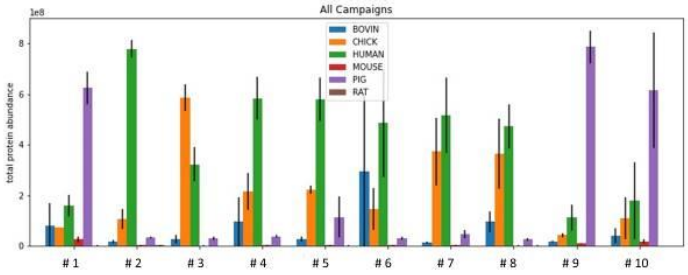
Fig. 1



P71

Mass spectrometry-based proteomics in the study of communication between ovarian cancer cells

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Due to the lack of effective treatment, the rate of mortality for ovarian cancer holds first place among the other gynecological cancers. Recurrence that occurs in 80% of all ovarian cancer cases is direct evidence of the emergence of tumor chemoresistance. Chemotherapy generates massive amounts of dying cancer cells. The phenomenon when exogenous signals from drug-stressed cancer cells accelerate the proliferation of remaining neighboring tumor cells is often observed in the clinic, but the underlying mechanisms remain unclear. Here we consider ascitic fluids as natural secretomes of ovarian cancer cells and present the comprehensive study of the changes in the ovarian cancer cell secretomes caused by chemotherapy *in vitro* and *ex vivo*. First, we compared the proteomic profiles of paired ovarian cancer ascites before and after chemotherapy, assessed their impact on matched primary cultures of ovarian cancer cells, and analyzed the phenotype of tumor cells residing in the ascites. We found that ascitic fluids after therapy were enriched with spliceosomal components and were able to promote chemoresistance and mesenchymal-like features of recipient patient-derived cells. We further confirmed this phenomenon on *in vitro* models of homogeneous cancer cell lines but we did not observe the same effect on normal human fibroblasts. Next, using fluorescence labeling and modified SILAC technology, we showed that under stress conditions, spliceosomal components are relocalized from the nucleus to the cytoplasm of ovarian cancer cells, exported to the extracellular space, and subsequently transported to recipient tumor cells. Finally, we showed that increased levels of several spliceosomal proteins contribute to therapy

resistance and partially recapitulated the effect of therapy-induced secretomes. We suggest that splicing factors circulating in the extracellular space can "prepare" intact cancer cells for subsequent drug treatment. Thus, understanding of the nature of these extracellular signaling networks could unravel the mechanism of tumor cell adaptation to treatment and increase the efficacy of standard chemotherapy.

The work was supported by the Russian Science Foundation project 19-75-10123 (LC-MS/MS analysis) and grant 075-15-2019-1669 from the Ministry of Science and Higher Education of the Russian Federation (RNA-seq analysis).

P72

Mass Spectrometry and multiomics approach to identify atabecestat-(metabolite) adducts and define pathways of atabecestat-(metabolite) T-cell activation

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Background

The orally administered BACE inhibitor atabecestat was developed to treat Alzheimer''s disease. However, clinical trials were halted due to drug induced liver injury (DILI) characterised by increased hepatic enzymes in patients. The aim of this study was to define pathways of drug-specific T-cell activation and identify the exact antigens that trigger T-cell responses using mass spectrometry based multiomics methods. A better understanding of the molecular mechanism of atabecestat DILI will assist to develop platforms for assessing intrinsic immunogenicity of novel chemical entities and safe drug design.

Methods

Atabecestat was incubated with metabolising systems (human liver microsomes, CYP3A4, HepG2), model proteins (HSA, GST-a1, GST-Pi), glutathione and N-Acetyl lysine. Drug metabolites formed in the metabolising systems were analysed by multiple mass spectrometry methods. Proteins were processed by Coomassie Blue 1D 10% acrylamide gels and tryptic digestion, followed by LC-MS/MS analysis. PBMC from healthy donors were cultured with atabecestat and diaminothiazine (DIAT) metabolites for T-cell cloning. Atabecestat and DIAT responsive clones were characterised by T-cell phenotype and pathways of T-cell activation.

Results

Multiple atabecestat-glutathione conjugates were detected in the presence of CYP3A4 by mass spectrometry analysis, indicating a thiol reactive intermediate was formed. Atabecestat was found to covalently bind to a lysine residue on GST-A1 in the presence of CYP3A4 and glutathione, but not to GST-Pi. This adduct was formed through cross-linking of a lysine residue and the thiol moiety of glutathione. Furthermore, a Schiff base adduct was formed with multiple lysine residues on HSA by a reactive atabecestat aldehyde metabolite. DIAT responsive CD4+ and CD8+ T-cell clones were generated which proliferated and secreted IFN-γ, in response to DIAT but did not cross-react with the parent drug. Activation of DIAT-responsive T-cell clones generated from healthy donors took place via direct interaction with MHC class I dependency.

Conclusion

The formation of a glutathione conjugate and covalent adducts with proteins was dependent on CYP3A4 metabolism. Identification of these drug protein adducts suggest potential *in vivo* activation of atabecestat T-cells in patients via the hapten mechanism. This, coupled with the *in vitro* generation of DIAT T-cell clones in patients and healthy donors suggest involvement of the adaptive immune system in atabecestat DILI.

P73

Proteome analysis of the serological response of cystic fibrosis patients to *Aspergillus fumigatus* protein antigens revealed possible diagnostic markers for Aspergillus infections

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Objectives: Cystic fibrosis (CF) is a genetic disease that causes thickened mucus and primarily affect the lungs. The human-pathogenic fungus *Aspergillus fumigatus* is one of the most frequently found molds that colonize the lung of these patients. Around 10% of patients develop an allergic bronchopulmonary aspergillosis (ABPA), which is characterized by a hypersensitivity response. The diagnosis of *A. fumigatus* lung colonization and ABPA is difficult due to a lack of a unique biomarker and standardized diagnostic criteria. To improve the diagnosis of *A. fumigatus* infection in CF patients, we aimed at screening for new *A. fumigatus* extracellular immunoreactive proteins with diagnostic value by using serological proteome analyses (SERPA).

Material & Methods: After TCA precipitation from culture supernatant, we separated extracellular proteins of *A. fumigatus* by 2D gel electrophoresis. Patient IgG antibodies reactive to *A. fumigatus* protein antigens were identified using multiplex fluorescent Western blotting with sera from CF patients with *A. fumigatus*-positive sputum cultures. Sera from patients with probably invasive aspergillosis (IA), CF patients without *A. fumigatus* diagnosis and healthy volunteers served as control groups. Antigenic maps were aligned to 2D-gels and antigenic spots of interest were identified by MALDI-TOF/TOF or LC-MS/MS. Decision tree models were used for the detection of *A. fumigatus* protein antigens with diagnostic potential. The analysis of these antigens is ongoing and will be tested by Western blots and indirect ELISAs using recombinantly produced antigens.

Results: In total, 44 extracellular protein antigens that gave a positive IgG antibody signal were identified, 22 of which were described as antigen for the first time. Several protein antigens showed a high diagnostic potential. In general, CF patient sera recognized a higher number of different protein antigens compared to control sera.

Conclusion: In summary, our comprehensive analysis detected new *A. fumigatus* antigens and provide possible new targets for diagnosis of *A. fumigatus* infections in CF patients.

P74

Proteasomal contribution to the MHC class I immunopeptidome deduced from identified immunopeptides

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Objective:

The immune system monitors the proteome of cells in order to detect intracellular pathogens. Therefore, cells present degradation products of proteins on their cell surface bound to MHC class I. Recognition of MHC class I-peptide complexes by $CD8^+$ T cells leads to T cell activation and, eventually, to cell death of the antigen presenting cell. Most immunopeptides are degradation products of the proteasome. While the C-termini of these peptides are not affected by the MHC class I

peptide loading, the N-termini are trimmed by aminopeptidases during peptide loading.

Interferon-γ (IFNγ) is a cytokine and induces expression of proteasomal subunits of the immunoproteasome a type of proteasome with slightly altered cleavage pattern compared to the constitutive proteasome. Furthermore, IFNγ enhances surface expression of MHC class I. In line with this, IFNγ has a tremendous influence on the immunopeptidome.

However, the influence of the different proteasomes on individual peptides presented by MHC class I has not been analyzed so far and the proteasomal contribution to the immunopeptidome has never been directly quantified. Therefore, we investigated the proteasomal origin of the immunopeptidome using the proteasomal cleavage prediction tool Pepsickle.

Method:

A549 cells were treated with IFNγ for 24h. Untreated A549 cells served as control. The cells were lysed and the MHC class I complexes were precipitated. Immunopeptides were purified and analyzed by mass spectrometry. The identified peptides were analyzed using the software MixMHCpred to identify peptides specific for expressed HLA alleles. Peptides with a binding rank ≥ 2% were discarded. Furthermore, only peptides repeatedly identified on the MS2 level were further processed. Annotated peptide sequences with eight N- and C-terminal amino acids were analyzed using the proteasomal cleavage score prediction tool Pepsickle.

Results:

Overall, we identified 5.286 peptides of which 1.012 MHC class I specific peptides passed the rigorous threshold for the cleavage score calculation. The median cleavage score of peptides C-terminally cleaved by the constitutive proteasome was 0.52315 for untreated cells and 0.617 for IFNγ-treated cells. The C-terminal cleavage score for processing by the immunoproteasome was 0.53885 for untreated cells, whereas IFNγ treated cells had a median cleavage score of 0.6555. For both proteasomes, the difference was very significant (p-value 0.0078 for the constitutive proteasome and p-value 0.002 for the immunoproteasome). The N-terminal cleavage score for the constitutive proteasome and the immunoproteasome was not significantly changed.

Conclusion:

We confirmed that enhanced activity of the immunoproteasome translates to an increased presentation of immunoproteasome-derived peptides on the cell surface by MHC class I. Furthermore, IFNγ also increases the MHC class I presentation of peptides generated by the constitutive proteasome

P76

Development of mass spectrometry-screening methods for quality assessment of Cryo-EM standard

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Objective

Cryo electron microscopy (Cryo-EM) determines biomolecular structures in near native state. As the success of EM grid preparation and obtaining a high-resolution structure strongly depends on the homogeneity and stability of the sample, quality control is crucial. Mass spectrometry (MS) provides the capability to screen biomolecules at relatively higher throughput and lower cost. Using MS to screen proteins prior to Cryo-EM imaging can thus accelerate structure elucidation and provide a much-needed quality assessment. Here, we developed Liquid Chromatography (LC)-MS-screening methods including native and intact protein assays for quality assessment of Cryo-EM standard, Apoferritin.

Methods

Recombinant human Apoferritin (~512 kDa, 24-mer) was expressed in *E. coli*. Other protein complexes were purchased from Millipore Sigma. Quality and stability of different batches of Apoferritin as well as other protein complexes was verified by SDS-PAGE, UV HPLC, and infusion or LC-MS using a Thermo Scientific™ Vanquish™ Flex UHPLC System coupled to a Thermo Scientific™ Q Exactive™ UHMR or a Thermo Scientific™ Orbitrap Eclipse™ mass spectrometer in native and denatured conditions. Data was analyzed using Thermo Scientific™ BioPharma Finder™ 4.1 Software.

Results

Understanding the quality of a Cryo-EM sample prior to microscopy is a critical step in achieving a high-resolution image. Recombinant Apoferritin 24 mer complex is widely used as a Cryo-EM standard. Critical attributes of such standards are: 1) subunit sequence confirmation; 2) correct complex assembly and homogeneity; 3) stability and aggregation. Recently, mass spectrometry emerged as a new technique to provide a solution to meet the new requirements for Cryo-EM sample screening, while significantly speeding up the sample preparation workflow. In this study, we used high resolution intact/top down analysis of dissociated subunits to confirm its sequence. Complex homogeneity and aggregation levels were analyzed by on-line buffer exchange (OBE)–LC-native MS method. Short desalting cartridge used for OBE serves as a good alternative for SEC. Pseudo MS3 (3) was performed to confirm subunits identity directly from protein complex.

Multiple batches of recombinant apoferritin prepared in different buffers and conditions were analyzed and compared with Cryo-EM data. First, our data demonstrates that native MS sample screening shows strong correlation to the quality of prepared Cryo-EM grids, both in terms of aggregation, particle distribution and stability. Secondly, the MS data helped to inform decision making on the biochemical preparations, for example by showing how increased levels of TEV protease increased the number of truncated subunits and correlated well with lower particle density in Cryo-EM.

Conclusion

These results clearly demonstrate the utility of native and intact LC/MS for Cryo-EM sample screening and sample preparation optimization for Cryo-EM structural studies.

P77

High throughput single-shot proteomics on the timsTOF Pro 2

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Objective

Rapid and in-depth proteomics have become a hallmark for the timsTOF platform powered by PASEF technology. TimsTOF Pro enables sensitive and accurate proteomic analysis from a variety of samples including cell lysates, tissues, pulldowns and body fluids in a robust fashion. Recently introduced timsTOF Pro 2 instrument offers all these benefits with improvements in downstream ion transfer from the TIMS cartridge and better robustness of the instrument combined with optimized methods to make better use of the hardware. Here we show the performance of the instrument to quantify cell line proteomes in different gradient lengths 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 prepared digests from HEK cell lines were used for benchmark measurements. Peptides were loaded on a Aurora-25 cm column using nanoELute coupled directly to a timsTOF Pro 2 instrument via a Captive Spray ionization source. 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 also processed offline in MaxQuant. For DIA, data were processed in Spectronaut. Data were filtered at a 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 gradient (80-minute runtime) injections. These runs typically resulted in identifying in the range of 6000 protein groups. Under similar conditions, using inhouse digested HEK peptides with an improved sensitive sample preparation protocol, we could quantify in the

range of 7000 protein groups using DDA methods. With DIA analysis, HEK peptides resulted in about 8000 protein groups and more than 70000 unique peptide sequences. Using the same platform, 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.

Conclusion

The timsTOF Pro 2 enables rapid and sensitive quantification of about 7000 protein groups in single-shot injections.

P78

Intercellular Transfer of Cell Surface Proteins by Cell-free Analysis Involving Chip-based Sensing Coupled to Proteomics: Identification of Glycosylphosphatidylinositol-Anchored Proteins and Regulation by the Metabolic State

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Question: Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are anchored at the surface of mammalian blood and tissue cells through a carboxy-terminal GPI glycolipid. Eventually, they are released into incubation medium *in vitro* and blood *in vivo* and subsequently inserted into neighboring cells potentially leading to inappropriate surface expression or lysis. To obtain first insight into the potential (patho)physiological relevance of intercellular GPI-AP transfer and its biochemical characterization, a cell-free chip- and microfluidic channel-based sensing system was introduced.

Methods: The major advantage of studying cellular processes with cell-free assays, in general, relies on the use of defined molecular components and experimental conditions as well as on their straightforward manipulation with the aim to identify the optimal configuration, which may also be relevant *in vivo*. For this, adipocyte or erythrocyte plasma membranes (PM) were covalently captured by the TiO2 chip surface operating as the acceptor PM. To measure transfer between PM, donor erythrocyte or adipocyte PM were injected into the channels of a flow chamber, incubated and washed out and the type and amount of proteins transferred to acceptor PM evaluated with specific antibodies. Antibody binding was detected as phase shift of horizontal surface acoustic waves propagating over the chip surface

Results: The data generated with the chip-based SAW sensing demonstrated that (i) rat and human adipocyte and erythrocyte PM can serve as both donor and acceptor for the transfer of GPI-APs, (ii) transmembrane proteins do not undergo transfer to any detectable extent, thus confirming previous findings, (iii) transfer efficacies differ between rat and human adipocyte and erythrocyte PM being highest between erythrocytes, (iv) both donor and acceptor PM determine transfer efficacy, compatible with release of GPI-APs from donor PM as well as their translocation into acceptor PM being rate-limiting for transfer, (v) transfer of GPI-APs is affected by the incubation conditions and the milieu surrounding the donor and acceptor PM with serum proteins downregulating its efficacy, (vi) interaction of the core glycan of the anchor of GPI-APs with serum proteins, such as GPLD1 (in particular in the inhibited state) or α-toxin, causes lowering of transfer efficacy, suggesting that this action mode mediates (part of) the inhibitory effect of serum proteins and (vii) transfer involves the incorporation of full-length, but not of anchor-less GPI-APs or transmembrane proteins, together with annexin-V and cholesterol into micelle-like complexes rather than into membrane-/vesicle-like or lipoprotein-like structures.

Conclusions: The novel chip-based sensing system may be useful for the prediction and stratification of metabolic diseases as well as elucidation of the putative role of intercellular transfer of cell surface proteins, such as GPI-APs, in (patho)physiological mechanisms.

Fig. 1

Fig. 1

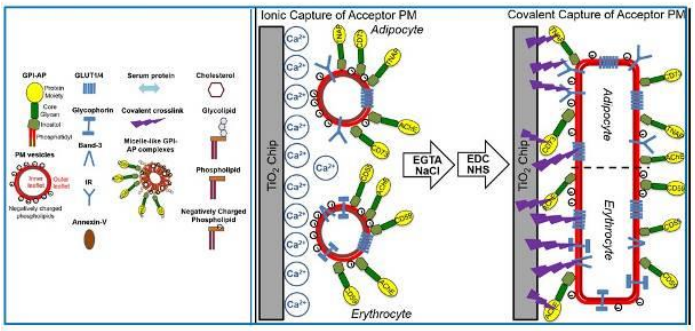
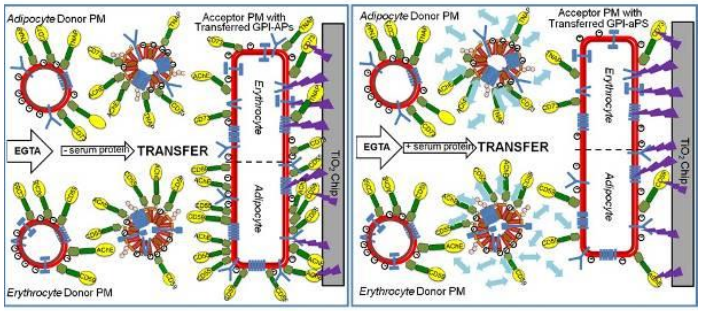


Fig. 2

Fig. 2



P79

Rapid & scalable off-line peptide fractionation on zwitterionic magnetic microparticles

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Fractionation of peptides is a common approach for reducing the complexity of digests and thus achieving deeper proteome coverage with bottom-up proteomics workflows. This is commonly done by employing chromatographic peptide separation via ion exchange, hydrophilic affinity and most often high-pH reverse phase. Although these methods are efficient in terms of peptide separation, they are also time-consuming, require auxiliary equipment such as liquid chromatography systems, and do not scale efficiently with input material. Conversely, magnetic microparticle based methods are quick to implement on the bench, allow for parallel sample processing, can be easily automated on common magnetic handling stations, and scale by simply adjusting digest amount to microparticle ratio. Here we evaluate the peptide fractionation efficiency of magnetic microparticles with zwitterionic functionality[JJ1] [SS2] . We tested the effects of various organic solvents and pH for hydrophilic affinity-based peptide separation. When a 100µg complex peptide sample, from a HEK digest, was applied [IG3] [IG4] to the magnetic microparticles followed by collection of six fractions (4 organic and 2 aqueous), we observed an increase of 40% and 30% respectively in peptide and protein identifications in comparison to the standard, non-fractionated sample (all data acquired using a 30 min gradient on a Dionex 3500 nanoRSLC coupled to a Sciex 6600 TTOF in DIA mode). High orthogonality was achieved particularly across organic and aqueous fractions. We are currently evaluating fractionation using pH in addition to testing the scalability of the workflow with lower input material. Finally, we would like to compare magnetic bead-based fractionation with the 'gold standard' high pH reverse phase method.

Fig. 1

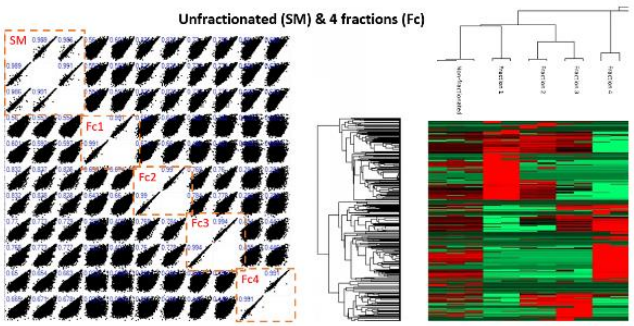
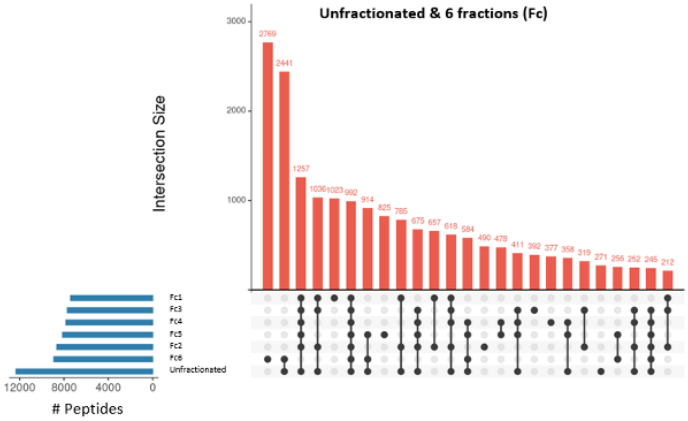


Fig. 2



P80

Application of a library-free dia-PASEF approach for high throughput and high sensitivity proteomics

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Objective

Data-independent acquisition (DIA) promises reproducible and accurate protein identification and quantification across large sample cohorts by using wide selection windows, rather than selecting individual peptides in DDA, to ensure that all precursor ions are fragmented in every sample. dia-PASEF (Meier et.al.,2019) takes advantage of the additional dimension of separation provided by trapped ion mobility (TIMS). The combination of DIA

with PASEF allows to compensate for the traditional DIA pitfalls: by using a pattern of m/z isolation windows within consecutive tims events, the percentage of ions used in the dia-PASEF can be greatly increased. The dia-PASEF cycle time can be reduced to make it compatible with short gradient separation while preserving a high selectivity. Here, we evaluate benefits of dia-PASEF including library-free data processing for very short gradients enabling ultra-high sample throughput proteomics. Moreover, we will demonstrate the performance of dia-PASEF on low sample loads down to 125 pg.

Methods

K562 tryptic digests (Promega) were analyzed by coupling EVOSEP One (EVOSEP) to a trapped ion mobility spectrometry – quadrupole time of flight mass spectrometer (timsTOF Pro 2 for short gradient data and timsTOF SCP for low sample loads) using optimized dia-PASEF schemes. Data was processed using library-free approach with DIA-NN 1.8 (Demichev et.al, 2021) incorporated into the PASER software (Bruker) and Spectronaut (Biognosys) using default settings.

Results

We used a 300 samples per day method (SPD, 4.8 min. run time) to evaluate the performance of dia-PASEF for high throughput proteomics. In classical DIA data analysis approaches spectral libraries are inevitable, being the basis for data extraction. Nowadays, also library-free approaches exist, resulting in increased throughput as there is no need for extensive fractionation and data acquisition using DDA. DIA-NN and Spectronaut both include software modules which enable operating without a spectral library. Using this approach, we identified 3600 protein groups / 23,500 peptides in just 4.8 min run time from 200 ng sample load.

To demonstrate the potential of dia-PASEF for high sensitivity proteomics we used the timsTOF SCP mass spectrometer in combination with low flow rate delivery from the Evosep system (Whisper100, 40 samples per day with gradient flow of 100nl/min). Using this setup, 1249 (±123, n=6) protein groups could be reproducibly identified from 125 pg K562 tryptic digest using dia-PASEF following the library-free based approach. To further increase proteome coverage for both, high throughput and high sensitivity proteomics, we will investigate additional new developments.

Conclusion

The presented results demonstrate the benefits of using dia-PASEF acquisition for high throughput and high sensitivity proteome studies using library-free approaches.

P81

Evaluation of prm-PASEF on a newly designed ultra-high sensitivity tims-Q-TOF

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Introduction

New acquisition strategies based on the use of Parallel Accumulation Serial Fragmentation (PASEF) or dia-PASEF approaches have improved the sensitivity and data completeness performances of untargeted proteomics strategies. Despite this development, targeted proteomics approach is still a reference methodology for the verification of biomarker candidates in large sample cohorts. It increases the sensitivity but also alleviates the problem of quantitative missing values between samples. prm-PASEF has therefore been developed to translate the advantages of the parallel accumulation serial fragmentation (PASEF) acquisition strategy to the targeted proteomics field. In comparison with standard SRM or PRM methods, the prm-PASEF increases the number of peptides that can be targeted in a single acquisition method, without compromising the selectivity or the sensitivity. In this communication, we evaluate the potential of this method when used in combination with an instrument designed for ultimate sensitivity.

Methods

Commercially available K562 cell line digest (Promega) was mixed with a mixture of isotopolog peptides (Pierce™ LC-MS/MS System Suitability Standard) containing 5 isotopolog for each of the 7 peptides in the mixture. Four solutions were prepared to cover a 25 fmol to 16 amol isotopolog concentration (Samples A1 and B1) or a 1 fmol to 0,7 amol isotopolog concentration (samples A2 and B2) in a 5ng (samples A1 and A2) or 100ng (samples B1 and B2) background

Samples have been injected in triplicates a on a 25cm X 75 µm pulled emitter column (IonOptics) and separated using a 30 min gradient generated by a nanoElute nano-HPLC system (Bruker) coupled to a standard timsTOF Pro 2 or to a newly designed ultra-high sensitivity timsTOF SCP (Bruker) operated in dia-PASEF or prm-PASEF mode. Data have been processed using Skyline daily.

Results

The prm-PASEF method was set up to target either the 35 isotopologs alone or in combination with 264 endogenous K562 peptides, and sample B1 was first measured on a timsTOF Pro 2. The co-isolation of the 264 endogenous peptides did not influence the intensity measured for the 35 isotopologs. In these conditions, more than 93% of the targeted peptides had a CV<20%, the median CV value being below 3%. Depending on the peptides, the Lower Limit of Quantitation (LLOQ) were comprised between 16,75 and 250 amol.

Using these results as a starting point, this communication will describe the outcome of similar measurements performed for samples A1, A2, B1 and B2 on the newly designed timsTOF SCP to evaluate and characterize its targeted proteomics performances. Those results will also be compared to what could be obtained from the same sample and gradient using an untargeted dia-PASEF approach.

P82

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

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Objective

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. Previously, we developed a multiplexed TMT11plex standard using three yeast knockout strains to assess co-isolation interference. Although this standard is useful for method optimization, 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.

Method

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. For liquid chromatography (LC), we used an Thermo Scientific™ Ultimate™ 3000 nanoLC in combination with an EASY-Spray C18 50cm column, with or without a FAIMS Pro™ Interface. Samples were analyzed on a Thermo Scientific™ Eclipse Orbitrap™ and Exploris™ 480 mass spectrometers. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 3.0 software using the SEQUEST® HT and COMET search engines.

Results

Proteomics standards are important tools used to optimize methods and assess LC-MS performance over time or across platforms. With the introduction of TMTpro 18plex reagents, we sought to develop new protype standards. We generated various prototype standards by mixing protein extracts from one or more yeast knockout strains with a wild type, parental strain. Unlike mixed proteome standards using protein extracts from different species, our standard is designed to keep same level of proteome complexity, minimize protein level abundance variability during initial, bulk protein extract measurements and mixing.

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.

Conclusion

Overall, we believe this new standard will enable the assessment and optimization of instrument performance for TMTpro isobaric tag-labeled, multiplex samples.

P83

Comparison of digestion methods supporting SDS depletion for Mass Spectrometry

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Efficient sample lysis for mass spectrometry covering the whole proteome is a continuous challenge. One of the common extraction method is the use of detergents like sodium dodecyl sulfate (SDS) which efficiently solubilizes proteins as well as lipids that form membranes. During protein digest, SDS has to be removed because of the incompatibility during liquid chromatography as well as electrospray ionization. Suspension trapping (S-Trap) is a commercially available filter, which assists the removal of SDS during the digestion process and offers a fast and robust sample preparation for MS. Our objective was to improve this method in terms of protein identification and evaluated the performance using other common digestion methods.

We compared varying protocols of S-Trap digests using different organic solvents as buffers and we adjusted the S-Trap protocol to our standard filter aided sample preparation (FASP) procedure. Additionally we confronted the improved S-Trap protocol with common digest methods including filter aided sample preparation (FASP), TCA precipitation, solid-phase-enhanced sample preparation (SP3) and the recently published SDS-cyclodextrin-assisted sample preparation (SCASP) method.

We proved that S-Trap efficiently removed SDS from extracts containing up to 5% SDS and that the use of acetonitrile (ACN) as loading and washing buffer performs similar like the specified methanol buffer. Protein identification rates could be increased by extended digestion over night as well as by avoiding the evaporation step after peptide elution. Additionally, the S-Trap digest compares well in terms of identification rates, coefficient of variation and missed cleavages achieved by FASP digest. SP3 did not remove SDS completely and the SCASP digest showed remaining cyclodextrins within the peptide solution. TCA precipitation was able to compete with S-Trap and FASP digests in terms of protein identifications but showed a slightly increased missed cleavage rate.

We conclude to have optimized the S-Trap digest in terms of identification rates as well as reduced health risks by using ACN instead of methanol. These findings might be of great interest for the MS community allowing an SDS-based sample preparation method, which is fast, simple and reproducible.

P84

Benchmark of Micro-flow Chromatograph for Robust Proteomics Analysis

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Liquid chromatography-mass spectrometry (LC-MS) has been a powerful analytical tool in protein identification and quantification. In the past, 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 Fisher ScientificTM NG micro-flow UHPLC System coupled to Thermo Fisher ScientificTM Orbitrap ExplorisTM 240 mass spectrometer. Gas-phase fractionation (GPF) was performed to improve protein and peptide coverage using Thermo Fisher ScientificTM High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) Pro Duo interface.

Different amounts of HeLa protein digest standard and TMT-11plex Yeast digest standard were loaded onto a PEPMAP C18 2um 150x1mm column, separated by 30min and 50min LC gradients and analyzed on Orbitrap Exploris 240 MS. LFQ analysis of digested PBMCs from a variety of animal species (human, mouse, rat, etc) were carried out at a high throughput of 24 samples/day to demonstrate the robustness over 100 injections. FAIMS Pro Duo interface was installed

to provide additional separations at different compensation voltages. Data was analyzed on Proteome DiscovererTM 3.0 software using MSPepSearch and CHIMERYS in parallel. Protein groups and peptide groups were filtered at 1% FDR rate.

Label-free proteomics performance on the micro-flow LC-MS setup was evaluated at 3 different loads of HeLa protein digest, 1ug, 5ug, 10ug, separated at both 30min and 50min gradient lengths on a 2 µm, 15 cm PepMap column. Each experiment condition was repeated for 5 injections in order to demonstrate the reproducibility. Data was processed in Proteome Discoverer 3.0 using MSPepSearch against the human NIST Orbitrap HCD library, in parallel with a new search engine, CHIMERYS. Percolator FDR calculation was used to only allow those spectra within 1% FDR rate to be reported. 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%). Multiplex quantitation analysis was carried out on the same setup introduced above, by injecting 5ug, 10ug and 20ug TMT 11plex Yeast digest. On average of 5 replicates, 90% of identified proteins and peptides were successfully quantified. Peripheral blood mononuclear cells (PBMCs) from a variety of animal species were purchased, followed by cell lysate and protein digestion. Digested peptides were fractioned by 6 compensation voltages and were acquired for triplicates on Orbitrap Exploris 240 MS.

This study demonstrates the outstanding performance and the broad range of proteomics applications of the micro-flow LC-MS workflow.

P85

The next-generation all-in-one nano-, capillary- and micro-flow LC system is paving the way for robust, fast, and deep LC-MS proteomics

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LC-MS technologies are becoming a standard in proteomics research and translational studies. The increased variety of LC-MS applications from single-cell proteomics to rapid sample profiling and requirements for long-term robustness, application versatility, system intelligence, and throughput capabilities push the technology developments. We present the next-generation all-in-one UHPLC system designed for nano-, capillary- and micro-flow high-sensitivity LC-MS applications.

We thoroughly evaluated the next-generation low-flow UHPLC system comprising the unique sample handling and sample separation technologies. The low-flow LC was coupled to Orbitrap Exploris 480 or TSQ Altis. The direct or trap-and-elute injection workflows for nano/cap and micro-flow separations (5-100 µL/min) on 75, 150, 300, and 1000 µm I.D. column ranging from 5 to 75 cm length were used to optimize separation conditions for typical tryptically digested protein samples.

We tested, optimized and pushed the performance limits for several key proteomics applications. In bottom-up proteomics we identified 7000 to 9000 proteins and > 85000 peptide groups with single-shot data-dependent acquisition by achieving ca. 20 s FWHM for 4 hours gradients using 75 µm x 75 cm long columns. We were able to improve results further by running 1.5 m long columns enabled by wide-flow pressure footprint with 1500 bar capabilities and active flow control. The high-throughput LC-MS methods for 15 cm nano and capillary columns allowed to analyze 180, 100, 60, 30, or 24 samples per day. The developed methods gave reproducible results with ca. 80% of peptides and proteins identified and quantified at three different locations. More than > 1500 injections and 6 months of continuous operation gave reproducible data for nanoLC separations proving the system robustness. Additionally, with micro-flow system capabilities and the throughput of up to 400 samples per day, the new low-flow UHPLC system enables targeted LC-MS screening in translational studies of large sample cohorts.

The novel design of LC hardware extends the boundaries of high-sensitivity LC-MS analysis in bottom-up proteomics

P86

The characterization and quantification of the cyclic peptide Oxytocin using Zeno CID and Zeno EAD

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Objective

The characterization and quantification of cyclic peptides using LC-MS/MS with CID represents a large challenge. Due to the nature of these molecules the number of fragment ions can be significantly higher than equivalent linear peptides, and often unselective for quantification in complex matrices. One of the challenges with quantification is finding selective fragment ions which will meet the analytical concentrations demanded with accuracy and precision. In CID typically large m/z fragments above the multiply charged precursor are chosen as they typically offer greatest selectivity in matrix. In this study we examine the potential of electron activated dissociation (EAD) for the quantification of the cyclic peptide Oxytocin. A unique workflow that fully leverages a Zeno trap coupled to a novel QTOF system in which >90% duty cycle is achieved in MS/MS mode is demonstrated and when coupled with MRMHR in either CID or EAD for quantification.

MATERIALS AND METHODS

Sample preparation: Oxytocin was prepared in protein precipitated rat plasma with 2:1 CH3CN: plasma and diluted 10-fold in water.

HPLC conditions: ExionLC AD system with a Phenomenex Kinetex XB-C18, 50x2.1mm, 1.8 µm column. Separation was performed over 2.15 minutes from 5% B to 99% B with a flow rate of 400 µl/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

MS/MS conditions: A SCIEX ZenoTOF 7600 system with Turbo V ion source and electrospray ionization (ESI) was used. The samples were analyzed in using Zeno CID MRMHR and Zeno EAD MRMHR. The TOF MS scan was scanned between *m/z* 100-1500, the CID and EAD MS/MS covering *m/z* 100 to 1400. EAD kinetic energy of 1eV and beam current of 5500nA.

RESULTS

The acquisition method for analyzing Oxytocin was setup to simultaneously monitor the TOF MS, EAD MS/MS and CID MS/MS. Fragmentation under CID and EAD conditions was optimized. In assessing the quantification performance of EAD, selectivity, limit of quantification, dynamic range, accuracy and precision were all compared to CID analyzing a dilution series from 0.25nM to 50nM with 6 replicates per concentration. The key difference between the CID and EAD calibration curves is the LLOQ achievable. With CID the LLOQ is limited by the precision of the low concentrations. For 0.5 and 1 nM the percent CV is over 30 % which is outside of regular bioanalytical criteria. For EAD the accuracy and precision is maintained down to 0.25 nM and linear to 50 nM. Percent CVs maintain good precision less than 9% and accuracy within 15%.

CONCLUSIONS

This work highlights the potential new avenue for both the characterization and quantification of cyclic or poorly fragmenting compounds. Electron activated dissociation (EAD) shows promise where poor reproducibility impacts the limit of quantification achievable. Further work will continue to explore across a wider range of cyclic molecules to understand how EAD can improve the analytical methodology.

P87

Leveraging a higher duty cycle DIA acquisition on a novel QTOF for enhanced proteomics analysis

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Objective

The ability to identify and quantify large number of proteins and peptides is of great importance in translational medicine and life science research. 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. Here we used a research version of ZenoTOF7600 system, equipped with a novel Zeno

trap, to evaluate gains in protein identifications at various HeLa peptide loads when using variable window Zeno SWATH relative to SWATH acquisition.

Materials and Methods

Sample Preparation: Lyophilized HeLa digest was purchased from Thermo Fisher Scientific and reconstituted with 95% buffer A (water with 0.1% formic acid) and 5% buffer B (acetonitrile with 0.1% formic acid).

HPLC: EvoSepOne (EvoSep, Denmark) had buffer A and buffer B as running solvents and operated in 200 SPD (samples per day, 5.6 min gradient), 100 SPD (11.5 min gradient), 60 SPD (21.0 min gradient) and 30 SPD (44.0 min gradient) throughputs.

MS Conditions: Research version of ZenoTOF7600 system was operated in SWATH and Zeno SWATH acquisition mode using the OptiFlowsource. 200 SPD, 100 SPD and 60 SPD was run in micro-flow configuration whereas 30 SPD was in the nano-flow configuration

Data processing: SWATH acquisition and Zeno SWATH data was processed using DIA-NN (v. 1.8) software

Results

When we utilize the Zeno trap to increase the duty cycle at the MS/MS level to over 90% in Zeno SWATH mode (Zeno trap turned ON), the quality of the MS/MS spectra increases greatly relative to that acquired in SWATH acquisition alone. Analyzing the same sample loads with Zeno SWATH rather than SWATH acquisition, we obtain 1.5-2.4x more protein group identifications at 20% CV threshold,1.8-3.2x more at 10% CV threshold and 1.2-1.5x increase in overall identifications at low (25-50 ng) protein loads at all SPD throughputs. At higher protein loads (200-500 ng), the increase in protein group identifications is 1.3-1.5x at 20% CV threshold and 1.4-1.8x at 10% CV threshold. With Zeno SWATH we are able to identify over 8400 protein groups for 200 ng and 500 ng HeLa load for the 30 SPD throughput with 80-90% of identifications at 20% CV, or 7600-7700 proteins at 60 SPD with comparable CVs. The "library-free" search approach for 500 ng load at 30 SPD identified 7700 protein groups, comparable number of IDs at CV thresholds relative to spectra-library approach and demonstrates the ease-of-use of the Zeno SWATH methodology in high-throughput proteomics.

CONCLUSIONS

Zeno SWATH enables a higher number of proteins identified and quantified (2-3x) especially at lower protein loads relative to SWATH acquisition

Zeno SWATH and library-free approach demonstrates the ease-of-use of DIA methodology for high-throughput proteomic workflows

P89

Reproducible and deep multi-site high-throughput LC-MS proteomic profiling of cell lysates and biofluids

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Throughput limitations and often preclude the adoption of nanoLC-MS methods for translational proteomics applications such as biomarker validation because the large sample cohorts need to be analyzed to reveal changes that stand out of biological, analytical, and sample preparation variation. The throughput (the number of samples per day) and MS utilization (the ratio of peptide elution window to total cycle time) can be boosted by utilizing LC instrumentation with the extended flow and pressure capabilities and minimal delay volumes.

Optimized LC-MS methods operated at flow rates in the range from 1.3 to 0.3 µL/min were created to maximize MS utilization (calculated as the ratio of peptide elution window to cycle time). The MS utilization gradually increased from 68% for the shortest method to 95% for the longest 60 min method. The length of the methods is also linked to the increased FWHM from ca. 3 sec for 8 min method to ca. 10 sec for 60 min method. To comprehensively estimate the analytical variability were compared the quantification and identification results collect (i) on the same low-flow LCMS instrument using the same separation column; (ii) on the same instrument using three separation columns of the same dimensions; (iii) on different instruments, with different separation and trap columns, located at three different laboratories in the US and Europe. The label-free quantification

showed that 72% of identified proteins have less than 25% variation of abundance for 100 continuous injections. The obtained results allow setting realistic estimates for abundance variation that are required to confidently detect targets above the multi-site analytical variation. We applied chimeric spectra data processing algorithm to results that improved the number of identified proteins and peptides from 120 to 50% for methods with total length from 8 to 60 min, correspondingly. Finally, we analyzed the set of crude serum samples for biomarkers discovery purposes with label-free quantification based on differences in cases and controls.

Fig. 1

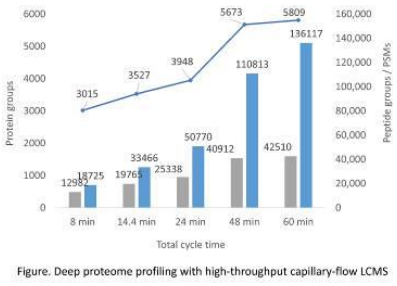

Figure. Deep proteome profiling with high-throughput capillary-flow LCMS

P90

Magnetic Bead Based Proximity Extension Assay for Sensitive Protein and Extracellular Vesicles Detection

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During the early stages of disease development, protein biomarkers can leak into the blood creating opportunities for early diagnosis of disease with minimally invasive sampling. These proteins biomarkers however are often masked by the presence of more abundant functional blood proteins, making specific detection a challenge with most current immune-assays, including the Proximity Extension Assay (PEA). Here, we report on the development of a magnetic bead based solid-phase PEA (SP-PEA) where target antigens are captured on antibody functionalized magnetic particles. Following capture, non-specifically bound proteins are washed off before PEA probes are added for detection of the bound proteins. Compared to solution phase-based PEA, SP-PEA admits the use of larger sample volumes to increase available target molecules, higher concentration of detection reagents for more efficient formation of detection complexes and washes for removal of nonspecific background. We compared SP-PEA to solution phase PEA for the detection of cytokines: interlukin-6, interlukin-2, interlukin-4, interlukin-10 and Tumor Necrosis Factor-alpha, and we demonstrated an increased sensitivity by 15 to 60 fold in buffer and chicken serum. We further expanded SP-PEA to detect extracellular vesicles (EVs) through combinations of proteins on the surface of specific EV populations.

Fig. 1

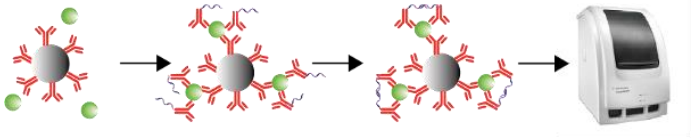
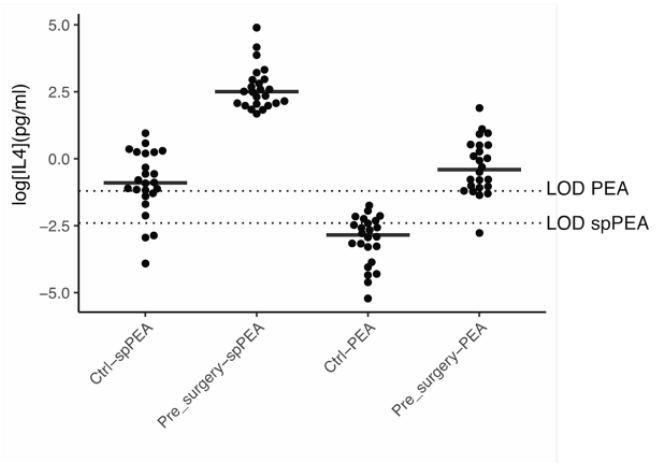


Fig. 2



P91

Proteomics and phosphoproteomics of molecular networks of stomatal immune responses

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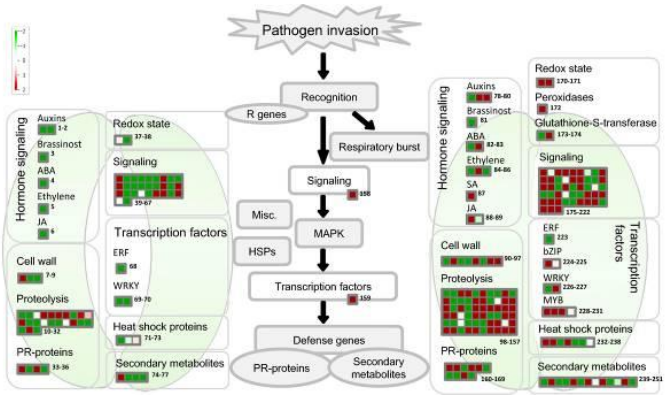
Stomatal openings represent a major route of pathogen entry into the plant, and plants have evolved mechanisms to regulate stomatal aperture as innate immune response against bacterial invasion. However, the mechanisms underlying stomatal immunity are not fully understood. Taking advantage of high-throughput liquid chromatography mass spectrometry (LC–MS), we

performed label-free proteomic and phosphoproteomic analyses of enriched guard cells in response to a bacterial pathogen Pseudomonas syringae pv. tomato (Pst) DC3000. In total, 495 proteins and 1229 phosphoproteins were identified as differentially regulated. These proteins are involved in a variety of signaling pathways, including abscisic acid and salicylic acid hormone signaling, calcium and reactive oxygen species signaling. We also showed that dynamic changes of phosphoprotein WRKY transcription factors may play a crucial role in regulating stomata movement in plant immunity. The identified proteins/phosphoproteins and the pathways form interactive molecular networks to regulate stomatal immunity. This study has provided new insights into the multifaceted mechanisms of stomatal immunity. The differential proteins and phosphoproteins are potential targets for engineering or breeding of crops for enhanced pathogen defense.

Fig. 1



P92

Dynamic light- and acetate-dependent regulation of the proteome and lysine acetylome in plants

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Question

In recent years, several global acetylome profiles have been reported in various organisms. The present study was conducted in the green alga *Chlamydomonas reinhardtii*, which is one of the most studied microorganisms in photosynthesis research and for biofuel production. Post-translational modifications (PTMs) can act as molecular switches for the control of protein function. Acetylation of the ε-amino group of lysine residues is a dynamic modification on proteins across organisms from all kingdoms. It is of great interest to understand PTMs and the underlying principles of metabolic pathway regulation by those modifications, especially in Chlamydomonas under different culturing conditions involving acetate as carbon source. The acetylome data, which we present here, provide new possible regulatory mechanisms based on changes of the posttranslational modification status on various metabolic enzymes.

Methods

We performed a mass spectrometry-based profiling of proteome and lysine acetylome dynamics in Chlamydomonas under varying growth conditions. Chlamydomonas liquid cultures were transferred from mixotrophic (light and acetate as carbon source) to heterotrophic (dark and acetate), or photoautotrophic (light only) conditions. Samples from these three different growth conditions were analysed in three biological replicates. Proteins were extracted, digested and free amino groups of peptides were labelled with stable isotope dimethyl-forms for quantification. Pooled peptides from the three growth conditions were subjected to pre-fractionation and enriched for lysine-acetylated (acK) peptides by immunoaffinity purification in addition to a full proteome analysis. All fractions were analysed by LC-MS. Proteins and acetylation sites were identified and quantified using MaxQuant.

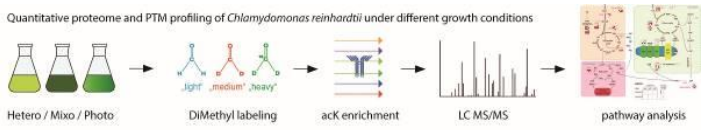
Results

In total, more than 5000 protein groups were identified with a protein FDR of < 1 %. The detected proteins have various functions in a broad variety of metabolic pathways. In addition, we identified almost 1400 acK sites in total. Out of the top20 enriched KEGG terms, fatty acid and propanoate metabolism, the pentose phosphate pathway, peroxisomal microbodies, photosynthesis, and antenna proteins are exclusively enriched as functional terms within the acetylated proteins.

Conclusions

Chlamydomonas proved to be an excellent organism to study PTM site regulation of proteins, which might be important for engineering algae and plant metabolism via genetic manipulations of their acK sites for lipid and biofuel production.

Fig. 1



P93

Optimization of protein extraction of *Arabidopsis thaliana*

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Effective protein extraction from biological samples is essential for proteomic analyses. High protein yield should not be to the detriment of labile posttranslational modifications such as phosphorylations.

Our goal was to optimize lysis conditions of the *Arabidopsis thaliana* in order to avoid losses at the phosphoproteome level alongside to preserve quality of proteome characterization.

Leaves of 5-weeks old plants were harvested and homogenized. SDT buffer (4% SDS, 100 mM DTT; 100 mM Tris-HCl, pH 7.6) was added to the fine plant powder and different lengths of the lysis and temperatures of the SDT buffer were tested. Filter-aided sample preparation (FASP) method was used for digestion of the proteins and 100 µg of each digest was subjected to a TiO2 phosphopeptide enrichment. Peptides and phopshopeptides mixtures were analysed by LC-MS/MS using Ultimate 3000 RSCLnano system connected to Thermo ScientificTM Exploris 480 mass spectrometer.

Extended duration of the lysis and higher temperature of the SDT buffer resulted in higher protein yields. However, LC-MS/MS analyses revealed that after application of 95°C SDT buffer the numbers of identified peptides and phosphopeptides were decreasing with prolonged time of the lysis. In case of lower lysis temperatures this decrease of identifications was reduced.

In summary, higher temperature and prolonged time of the lysis leads to increased proteins yields, on the other hand these conditions reduce counts of identified peptides and phosphopeptides.

This research was funded by European Regional Development Fund—Project "SINGING PLANT" (No. CZ.02.1.01/0.0/0.0/16_026/0008446). CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2018127, is gratefully acknowledged for the financial support of the measurements at the CEITEC Proteomics Core Facility.

P94

Reproducible identification and quantitation of protein carbonylation sites in human plasma

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[2]*Biotechnologisch-Biomedizinisches Zentrum, Bioanalytik, Leipzig, Germany*

Question: Develop a strategy for sample preparation and data analysis that allows the reproducible (i) identification of carbonylation sites as oxidative post-translational modification (PTMs) in human plasma proteins and (ii) quantitation of accordingly modified peptides.

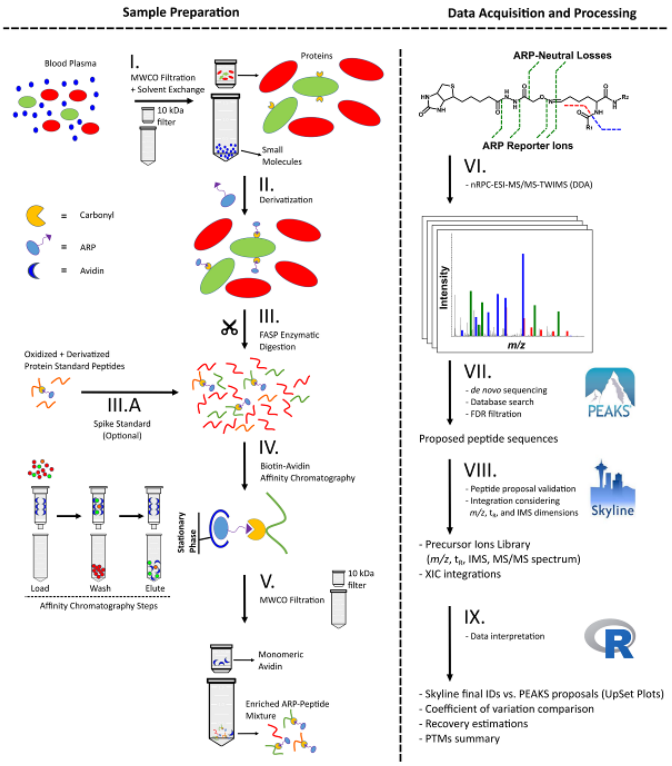
Methods: Oxidized human serum albumin (OxHSA) and human plasma proteins were derivatized with a carbonyl-specific biotinylated derivatization tag (aldehyde reactive probe; ARP) prior to enzymatic digestion. Non-derivatized peptides were depleted using avidin-biotin affinity chromatography prior to nano-reversed-phase chromatography coupled online to an electrospray ionization tandem mass spectrometer containing also a travelling wave ion mobility spectrometer (nRPC-ESI-MS/MS-TWIMS). Spectra were acquired in data dependent acquisition (DDA) mode and processed by combining de novo sequencing and database open mass searching (OMS) to identify known and novel carbonylation

modifications. Reproducible peptide chromatographic features were visualized in Skyline.

Results: A total of 462 ARP-derivatized peptides derived were identified including 239 detected in-vivo and 223 unique in OxHSA model. Carbonylation sites present in-vivo originating from reactive carbonyl species (RCS) localized at six different lysine modification hotspots (Lys10, Lys190, Lys199, Lys281, Lys432, and Lys525 of mature HSA) of which Lys190, Lys199, and Lys525 have been identified previously as preferential RCS targets. Modifications localized at Gln/Asn- and Glu/Asp-residues, proposedly forming imide and isoimide PTMs, were identified as abundant unspecific derivatization byproducts that can only be resolved by LC-MS/MS. Reported and new modification-specific reporter ions identified for ARP-derivatized peptide were essential for increasing the identification confidence of carbonylated peptides. Upscaling the initial protein contents from 0.2 mg to 2.0 mg increased ARP-peptides peak areas on average by 7.3-fold in the affinity-enriched samples. From the 239 ARP-peptides detected in-vivo, 68% and 76% had a coefficient of variation (CV) below 20% for the 0.2 and 2.0 mg sample replicates, respectively.

Conclusions: The easy and inexpensive strategy established to generate OxHSA yielded diverse carbonylated peptides that can be used for intra-batch quality control. Increasing the initial protein content increased the quantity of derivatized carbonylated peptides trapped in affinity chromatography leading to an improved sensitivity in the detection and quantitation of carbonylation sites in human plasma. Although the current study did not address the biological relevance of the identified carbonylated proteins and individual carbonylation sites, the baseline detection of derivatized carbonylated peptides from plasma of healthy individuals provides a valid base for further quantitative studies on cohorts in the context of oxidative stress related diseases where carbonylation sites are presumably very abundant.

Fig. 1



Schematic presentation of the analytical workflow applied to plasma samples for enriching ARP-derivatized peptides (left) and the consecutive LC-MS-based analysis from acquisition to data processing (right). Ultrafiltration of blood plasma was applied to remove small molecules and to reconstitute the sample in acidic conditions (i) to derivatize carbonylated proteins with ARP (ii). Proteins were digested with trypsin using FASP (iii) and the resulting peptide mixture was split and either mixed with an ARP-labelled digest of a model protein (iii.a) or directly enriched by avidin affinity chromatography (iv). After ultrafiltration to remove interfering monomeric avidin (v) the samples were analyzed by nRPC- ESI-MS/MS-TWIMS in DDA mode (vi). The generated tandem mass spectra were processed with a hybrid de novo and database search approach (vii) considering ARP-specific fragmentation patterns. All proposed ARP-derivatized peptides were validated by manual annotation of the mass spectra considering both drift times in IMS and retention times RPC (viii). The filtered peptide list and corresponding peak areas were further processed (ix) to assess recovery, sample preparation workflow reproducibility, and the location of the protein carbonylation site. Figure adapted from DOI: 10.3390/antiox10030369.

P95

Determination of glycation rates of blood proteins in an exotic model: the zebra finch

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Objective

Glycation is the non-enzymatic covalent bonding between reducing sugars and amino groups, resulting in the formation of Amadori compounds and advanced glycation end products (AGEs).[1] The adverse effects of abnormal protein glycation levels, e.g. when glycaemia is high, are known to promote ageing. Birds generally demonstrate slow ageing and are the vertebrates with the highest blood glucose levels. To gain knowledge on this bird paradox, we determined the relative abundance of the glycated forms of main blood proteins in zebra finch plasma and red blood cells

(RBC), and we analysed bird haemoglobin oligomeric state.

Method

Human and bird plasma samples and RBC were diluted in acidified water and analysed using LC-MS analysis (Agilent 1200 HPLC system equipped with a C8 column [vydac, GE Healthcare] coupled to a Bruker Maxis II Q-TOF). Data were treated using Data Analysis (v4.3). Glycation rates for bird plasma albumin, carbonic anhydrase, and serotransferrin and for bird and human haemoglobin (HB) in RBC were evaluated from the extracted ion chromatograms of the 10 most intense ions of the native and glycated forms. A similar analysis was performed for bird and human HB from hemolysates incubated in vitro for 3 days in a 30mM glucose solution. The bird HB structure was also studied by native SEC-MS analysis (Acquity UPLC H-Class system coupled with a Synapt G2 HDMS Q-TOF; Waters).

Results

In birds, several forms of the three plasma proteins we followed were detected, from non-glycated to triply glycated forms (rates ranging 11-50%). In human RBC, HB glycation rate ranged 5-12%, however, no glycated form of bird HB was detected (Fig 1 : Deconvoluted spectra of the haemoglobin β-chain in humans and zebra finches).

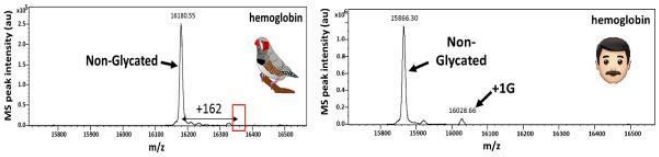
After incubation of RBC under forced glycation conditions, HB glycation levels rose quickly and gradually for human samples, whereas glycation of the bird protein was delayed and less pronounced. Moreover, SEC-MS highlighted the unusual presence of three different HB tetramer populations with balanced proportions and a still bound putative cofactor.

Conclusion

Our method successfully allowed the glycation rates of plasma proteins to be estimated. The absence of any glycated form for bird HB in vivo, together with the delayed low glycation rates of bird compared to human HB in vitro may reflect protection mechanisms specific to nucleated bird RBC, including peculiar structural features. To go further, we will investigate other avian species in order to have a comprehensive panel to correlate with phylogeny, feeding habits, and lifespan. This study illustrates of how MS analysis applied to non-standard model may help better decipher interrelationships between glucose metabolism and ageing.

1. Soboleva, A., et al., *Maillard Proteomics: Opening New Pages.* International Journal of Molecular Sciences, 2017. **18**(12): p. 2677.

Fig. 1



P96

The human macrophage capping protein CAPG is redox sensitive and controls the migration behavior of glioblastoma cells

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Objective

The amount of the macrophage capping protein CAPG has been described to be associated with progression and treatment response of several cancer types including glioblastoma. In previous work, we found CAPG as a potentially redox regulated protein. Here, we characterized potential redox-switches of CAPG and their functional consequences on glioblastoma cells.

Methods

In order to characterize potentially oxidized cysteine residues, we used PEG-switch assays, differential labeling of cysteine with isotope coded alkylating agents with subsequent mass spectrometric quantification of cysteine containing peptides. Furthermore, functional assays have been carried out including action polymerization assays, scratch assays and microscopy. Redox-state dependent interaction partners of CAPG were identified by affinity purification mass spectrometry.

Results

We identified two cysteines C282 and C290 as reversibly oxidized. Furthermore, we found a redox-dependent protein translocation of CAPG from the nucleus to the cytosol. This localization shift was also found for the newly identified CAPG interaction partner RAVER1. We hypothesize that both proteins might affect cell migration by modulating cell adhesion properties.

Conclusion

We provide strong evidence that not only the amount of CAPG is important for affecting the migration properties of glioblastoma cells but also a redox switch within CAPG including C282 and C290.

P97

Plant histone sample preparation for mass spectrometry using FASP and SP3 beads

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Introduction

Sample preparation of plant histones for mass spectrometry is complicated due to plant-specific (and even plant species-specific) contaminants which are present within the sample matrix. Efficient removal of these contaminants is necessary to obtain quality results from LC-MS/MS analysis enabling reliable quantification of histone post-translational modifications.

Objectives

Comparison of sample preparation of plant histones using SP3 (single pot, solid-phase-enhanced sample preparation on carboxylated paramagnetic beads) and FASP (filter-aided sample preparation).

Materials and methods

Nuclei from *Arabidopsis thaliana* were isolated using Percoll gradient followed by histone extraction into sulfuric acid. Filter unit or SP3 beads were used for histone preparation, including contaminants removal, derivatization at the protein level and digestion with trypsin. In-solution chemical derivatization of histone peptides and peptide purification step using SpinTips prior to LC-MS/MS was performed using the same protocols for both methods.

The peptides were analyzed by LC-MS/MS using Orbitrap Fusion Lumos Tribrid, and peaks of peptide precursors were quantified in Skyline software.

Results

Peptides originating from Arabidopsis histone extract were quantified to evaluate the impact of sample preparation on histone purity, the efficiency of chemical derivatization of amine groups, and the representation of post-translationally modified forms.

Conclusion

SP3-based approach substantially outweighed FASP-procedure regarding the time needed for sample preparation while keeping comparable performance in terms of peptide separation, identification, and subsequent quantification of post-translationally modified histone peptides.

This research was funded by European Regional Development Fund—Project "SINGING PLANT" (No. CZ.02.1.01/0.0/0.0/16_026/0008446). CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2018127, is gratefully acknowledged for the financial support of the measurements at the CEITEC Proteomics Core Facility.

P98

Phosphoproteomics response of *Aspergillus fumigatus* in adaptation to hypoxia

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1. Objective

The filamentous fungus Aspergillus fumigatus is an opportunistic human pathogen, which can cause life-threatening invasive pulmonary aspergillosis in the

immunocompromised host. The ability of the fungus to sense and adapt to low environmental oxygen concentrations is an important virulence trait with regard to invasive infections. In order to study the adaptational response on the protein and posttranslational level, we performed a quantitative proteomics and phosphoproteomics analysis by comparing fungal replicates grown under either normoxic or hypoxic (0.2% O_2) conditions in an oxygen-controlled fermenter.

2. Methods

Proteins were isolated from each three biological replicates of A. fumigatus grown under either hypoxia or normoxia by filtration, cryogenic grinding, solubilization in denaturing lysis buffer and trichloroacetic acid precipitation. In-solution digestion of each 6 mg protein was performed overnight with Trypsin/LysC at 37°C. Each 0.2 mg digested aliquots were directly used for the proteome analysis and each 5.8 mg of digested proteins were further enriched for phosphopeptides based on a TiO2/ZrO2 solid phase extraction protocol. All samples were analyzed in triplicates by nanoLC-MS/MS (Thermo QExactive HF). Proteome Discoverer 2.4 (PD2.4) and the search algorithms of Mascot 2.4, Sequest HT, MS Amanda 2.0 and MS Fragger 3.2 were applied to identify peptides and proteins. The ptmRS algorithm was used to calculate phosphosite probabilities and the Minora algorithm was used to perform label-free quantification. Phosphopeptide abundance ratios (hypoxia versus normoxia) were corrected against master protein abundance ratios of the shotgun proteome analysis.

3. Results

We identified 5136 proteins in total of which 2541 proteins carried at least 1 phosphorylation. Moreover, 10805 phosphopeptides with 10353 unique phosphosites were identified among all sample groups. By application of strict threshold values (fold change >4, ratio-adjusted p-value <0.05) we found 318 proteins and 1674 phosphopeptides of significantly different abundance (hypoxia versus normoxia). Functional enrichment (FunCAT, GO) of the significantly different proteins identified an involvement in oxidation-reduction processes and alcohol dehydrogenase activity. Significantly different phosphopeptides were derived from proteins functionally enriched in transcriptional control, DNA confirmation modification, and GTPase signaling. Interestingly, we found highly increased phosphorylation of autophagy-related proteins Atg1 (2988-fold at S621) and Atg13 (11324-fold at S852), a target of TOR and part of the autophagy-initiating Atg1 complex.

4. Conclusion

Phosphoproteomics of A. fumigatus revealed an involvement of autophagy under hypoxic growth conditions.

P99

Development of Phosphoproteomic Workflow for the Analysis of FFPE Tissue Sections

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Objective For efficient investigation of phosphorylation of small amounts of complex protein mixtures, it is essential to perform purification and enrichment steps before analysis. Our goal was to develop and optimize these sample preparation methods, and to apply them effectively to small tissue samples.

Methods Commercial HeLa cell line tryptic digest was used for the optimization of desalting and phosphopeptide enrichment steps. The developed methods were applied for the analysis of formalin-fixed paraffin-embedded (FFPE) lung, prostate and breast cancer and tumour adjacent normal tissue sections. On surface proteolytic digestion, reversed-phase solid-phase extraction with Pierce C18 spin columns, and phosphopeptide enrichment method were performed. Peptide and protein identification of the reversed-phase nanoHPLC-MS/MS measurements was performed using Byonic, and the label free quantification with SkyLine.

Results The developed phosphopeptide enrichment method (pipette tip-based TiO2 stationary phase, 50 mM citric acid / 1.5% TFA content in loading buffer) showed outstanding results for phosphopeptide enrichment from samples containing 500 nanograms of HeLa tryptic digest. With this citric acid enrichment method, reproducible identification and quantification of phosphopeptides can be obtained.

We optimized the composition of the sample loading buffer in order to reduce sample loss during the purification process. Using 0.1% heptafluorobutyric acid in water, the sample loss was reduced by ~30% compared to the manufacturer"s protocol.

The developed methodology was applied to FFPE tissue samples. Significant differences in phosphopeptides were identified between lung, prostate and breast adenocarcinoma samples.

Conclusion We have successfully developed and optimized sample preparation methods to study the phosphorylation of proteins in limited size samples. The developed workflow is suitable for the examination of phosphorylation in small lung, prostate and breast tissue sections.

Acknowledgements The research program was supported by the National Research, Development and Innovation Office: 2018-1.2.1-NKP-2018-00005 and FK131603.

P100

Isobaric labeling mass spectrometry to monitor ubiquitination dynamics upon proteasome modulation by small molecule inhibitors

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Proteins are tagged with the small protein ubiquitin to target them for degradation by the proteasome. Malfunctioning of the ubiquitin–proteasome system (UPS) leads to proteome imbalance and – thus – to cancer and neurodegenerative disorders. Since malignant cells have a higher dependency on the UPS compared to normal cells, proteasome inhibitors such as Bortezomib are used in the clinic for the treatment of multiple myeloma. Using quantitative isobaric labeling mass spectrometry in combination with ubiquitination enrichment technologies, we can monitor the dynamics of the ubiquitinome upon proteasome inhibition in great detail. We use this technology to study the modes of action of different (second generation) proteasome inhibitors.

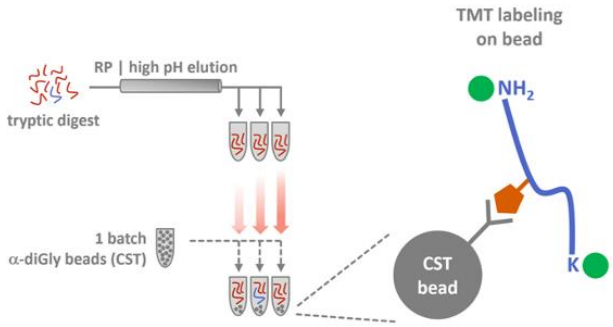
A quantitative mass spectrometry approach was based on SILAC and TMT labeling combined with diGly peptide enrichment. First, specific proteasome modules were selectively inactivated by RNAi knockdown or CRISPR/Cas knockout or by small molecule inhibitors. Next, the effects of several different proteasome inhibitors on the ubiquitinome were explored by combining peptide enrichment with isobaric (TMT) labeling over time. Finally, we have explored the added value of ion mobility mass spectrometry to the analysis of TMT labeled enriched modified peptides.

First, we developed an improved workflow for the enrichment and detection of diGly peptides that originate from ubiquitinated proteins upon tryptic digestion. Using a combination of crude peptide fractionation, optimized use of diGly antibody beads and an efficient peptide fragmentation regime, we were able to routinely identify >23,000 diGly peptides from a single sample. We then perturbed proteasome activity by selective depletion of subunits or by small molecule inhibitors. Malfunctioning of the proteasome resulted in a largely affected proteome, characteristic for changes in stress response, cell cycle regulation, apoptosis and the UPS. The effects were even more pronounced for the ubiquitinome, which was dramatically remodeled upon proteasome modulation. Although the far majority of proteins became increasingly ubiquitinated, many proteins showed heterogeneous ubiquitination patterns on different lysine residues. In addition, we selectively depleted the three proteasome associated deubiquitinases (DUBs) and observed remarkably differences in proteome and ubiquitinome remodeling, suggesting unique targeting specificities. Next, several small molecule DUB inhibitors such as Bortezomib and b-AP15 were used to target the proteasome in a specific manner and we used SILAC quantitation to monitor the deep ubiquitinome. To characterize ubiquitination profiles over time, isobaric labeling was used in a multiplexed fashion. This strategy allowed us to accurately measure and detect subtle changes of the ubiquitinome.

In conclusion, multiplexed quantitative proteomics combined with enrichment strategies allows for the monitoring of ubiquitination dynamics over time.

Poster presentation

Fig. 1



P101

Optimization of a high-throughput phosphoproteomics workflow on complex proteomes

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Objective: Despite its widespread use in laboratories, phosphoproteomics still faces many challenges. In particular, sample complexity and the dynamic range of peptide concentration are major limitations. Phosphopeptides are present in very low abundance disadvantaging their identification. One other difficulty of phosphoproteomics is its reproducibility, which is highly sample preparation dependent and largely influenced by the phosphopeptides enrichment step. Finally, the identification and quantification of posttranslational modifications (PTMs) are challenging for both MS acquisition and data interpretation. Indeed, finely tuned data treatment workflows to properly filter the large output of phosphoproteomics experiments and precisely localize the phosphosites are still required. In this work, our aim was to setup a complete workflow for phosphoproteomics experiments, from sample preparation to data treatment, in order to correctly identify, quantify and localize phosphosites in complex samples.

Method: Starting from tissue samples, three different extraction buffers were compared, with or without a precipitation step, resulting in six different protocols. After comparing all of them in terms of proteins and peptides identifications results, we kept the two best protein extraction protocols. An automated a phosphopeptides enrichment step was setup on those two protocols, using IMAC (Immobilized Metal Affinity Chromatography) cartridges on an AssayMAP Bravo (Agilent Technologies). The phosphoenriched peptide fractions were analysed on three different nanoLC-MS/MS platforms, namely a nanoLC-Q-TOF (TimsTOF Pro from Bruker Daltonics), a nanoLC-Q-Orbitrap (Q-Exactive HFX from Thermo Fisher Scientific) and a nanoLC-Orbitrap Tribrid platform (Orbitrap Eclipse from Thermo Fisher Scientific). Finally, different software tools were evaluated for data analysis including MaxQuant (Max Planck Institute of Biochemistry), Proline Studio (ProFI Proteomics) and Proteome DiscovererTM (Thermo Fisher Scientific).

Results: The two protocols displaying the best identification results before IMAC were (i) Protein extraction with 6M urea, 2M thiourea, 0.1M ammonium bicarbonate lysis buffer followed by classical in solution digestion and SPE; (ii) Laemmli lysis followed by a SP3 (Single-Pot, Solid-Phase-enhanced Sample Preparation) digestion protocol. After enrichment, the performances of the different sample preparations, MS acquisition methods and data treatment workflows were benchmarked and compared in terms of numbers of identified phosphopeptides and numbers of validated phosphosites.

Conclusion: We were able to optimize a complete phosphoproteomic workflow, from sample preparation to data treatment. This optimized protocol was then applied on different complex samples, namely mouse brain tissues and human cerebrospinal fluid.

P103

Analysis of mTOR pathway signalling in antibody-producing CHO cells using a bead-based western blot

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Chinese hamster ovary (CHO) cell lines represent the most commonly used mammalian cells for production of biopharmaceuticals. A profound understanding of signalling in different CHO cells can help to perform guided cell engineering, leading to higher production

yields in low producer cell lines. The detailed phosphorylation and expression analysis of key-players in the mechanistic target of rapamycin (mTOR) pathway is a prerequisite for the profound characterization of this prominent pathway influencing protein synthesis. The DigiWest (Treindl *et al*, 2016, Nat Commun 7,12852), a bead-based multiplexed western blot approach, is a useful tool for screening mTOR pathway activities in cell lines showing different productivity of recombinant proteins. The results could lay the base for target-oriented cell engineering.

We present a focused proteome and phosphoproteome profiling to measure mTOR signalling using the DigiWest approach. The novel method permits the performance of hundreds of Western Blots simultaneously in a multiplexed bead-based assay system. Proteins and their post-translational modifications (PTMs) can be detected and quantified by antigen-antibody reaction on magnetic beads. As there is a multitude of commercially available phospho-specific antibodies, this multiplexed assay format allows a closer look at signalling bottlenecks in production cells, while sample amount and analysis time can be minimized.

We present data on expression and phosphorylation of the members of the mTOR pathway in different antibody producing cell lines. We screened for commercial antibodies from different species for their applicability and identified 144 antibodies, which detect proteins or PTMs in CHO proteins. A mapping of the members of the mTOR pathway and their phosphorylation status was established, which showed differences in phosphorylation in antibody-producing cells downstream of the mechanistic target of rapamycin complex 1 (mTORC1).

Analysis of functional signalling changes for relevant members of the mTOR pathway can be performed by focused phosphor proteome analysis with antibody-based multiplexed assays. This should enable the comparison of different producer cells for biotherapeutics. The assay helps to gain a better understanding of molecular processes in CHO cells and can favor focused cell engineering in biopharmaceutical industry in the future.

P104

Discovery phosphoproteomics using electron-activated dissociation and high sensitivity on a novel QTOF system

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Objective

Phosphorylation is a key post-translational modification for understanding of biological signaling and plays important role in cellular senescence. Precise mapping of site-specific phosphorylation is the key to understand biology of senescence. While collision induced dissociation (CID) is fast and efficient, electron-based dissociation produces phosphopeptide fragments that retain CID-labile phosphorylation modifications. A new QTOF platform combines an electron activated dissociation (EAD) reaction cell and a Zeno trap for increased MS/MS sensitivity. This EAD cell produces efficient fragmentation with reaction time of 10-20 milliseconds, enabling data dependent acquisition on an LC timescale.

Material and methods

Sample preparation: Stable IMR90 human fibroblast that express the inducible ER:RASG12V upon (Z)-4-hydroxytamoxifen (4-OHT), were treated with 100 nM 4-OHT to induce senescence. Control cells were treated with methanol. Nuclei were purified followed by the cell lysis. Proteins were digested using trypsin/LysC proteases and phosphopeptides are enriched using the polymer-based metal-ion affinity capture (PolyMAC) spin tip (Tymora Analytical, USA).

LC-MS/MS conditions: Microflow chromatography was performed using a Waters ACQUITY UPLC M-class system in trap-elute mode with Phenomenex Kinetex 2.6 mm XB-C18 100 Å, 150 x 0.3 µm LC column. A 45 min gradient was used with a flow rate of 5 µL/min for all experiments. All mass spectrometry data was acquired using the ZenoTOF 7600 system (SCIEX). Data was acquired in data dependent mode for peptide workflows using either collision-induced activation or electron-activated dissociation. Zeno trap was activated in all DDA experiments.

RESULTS

Tryptic peptides from non-enriched samples were identified using either CID or EAD for fragmentation, resulting in about 39% identified by both approaches. Due to the higher frequency of MS/MS sampling using CID (~50 Hz), the overall number of unique peptide identifications was about 2-fold greater than when using EAD (~20 Hz).

Phosphopeptide-enriched samples of both control and treated samples were analyzed by LC-MS/MS using either CID or EAD. While CID produced the larger number of phosphopeptide identifications, the localization of the phosphorylation site is more confident based on the EAD data. A number of phosphorylated proteins were identified uniquely by EAD in the treated (D6-4OHT) cells. These initial results provide potential targets for further investigation into their role in oncogene induced senescence.

CONCLUSIONS

As demonstrated in this study, phosphopeptides can now be monitored using fast and efficient electron-based fragmentation. While not as fast for global proteome discovery, electron-based fragmentation on an LC-timescale is demonstrated. Based on these initial experiments, EAD provides complementary information to CID for phosphopeptide identification and potentially more robust localization information.

P105

SureQuantTM 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 EasyPepTM technology, phosphopeptide enrichment, validated multipathway AQUATM heavy-labeled phosphopeptide standards, and SureQuantTM targeted MS to quantitate changes in phosphorylated protein abundance across multiple stimulated cell lines. Specific phosphopeptide standards were chosen representing phosphosites from several different pathways including EGFR/HER, RAS-MAPK, PI3K/AKT/mTOR, AMPK, death and apoptosis, and stress (p38/SAPK/JNK) signaling. This novel workflow enables targeted quantitation of biologically relevant phosphorylation sites with high accuracy, precision, and specificity.

Methods

Multiple cell lines (MCF7/HCT116/A431/LNCAP/HepG2) cells were grown with different stimulation conditions (hIGF-1/hEFG) 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 ScientificTM PierceTM 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. To ensure optimal measurement for targets, a novel SureQuant targeted acquisition method was performed where real-time heavy peptides detection triggered high-sensitivity measurement of endogenous targets. Data analysis was performed with Proteome Discoverer and Skyline software.

Preliminary Data

In this study, we developed a complete workflow solution for targeted phosphopeptide analysis by combining EasyPep MS sample prep kits and SureQuant MS assay panel to quantitate biologically relevant signaling pathway proteins. Our optimized workflow combines Fe-NTA enrichment with 131 AQUA heavy-isotope phosphopeptide standards to monitor multi-pathway signaling. 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.

Novel Aspect

SureQuant multipathway phosphopeptide standard with novel SureQuant MS analysis allows reproducible, routine, and simultaneous quantitation of functionally relevant phosphorylation sites.

P107

In-gel methanol-d4 labelling strategy helps to identify a new class of D/E O-methyl (O-me) modifications present in the acidic patch of histones

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Objective

Protein D-Methylation was first described as a repair mechanism for damaged proteins (e.g. histone H2B) catalyzed by Protein L-isoaspartyl methyltransferase (Zhu, JBC 2006). In this context, we identified O-me of histone H4 at D24 (Biterge, Scientific Reports 2014). Here, we set out to describe O-me as a more general histone PTM in human cells. To differentiate endogenous methylation from artefacts introduced post cell lysis, we applied an in-gel methanol-d4 isotope labeling strategy for D/E residues susceptible to methyl-esterification.

Methods

MeOH adducts at D/E residues and peptide C-termini are known GeLC-MS artefacts. In order to distinguish them from endogenous O-me sites, we employed methanol-d4 during SDS gel fixation and staining steps, thereby introducing methyl-d3 esterification of D/E residues. All subsequent sample preparation steps were carried out in absence of primary alcohols (using ACN). Good sequence coverage was achieved by combined data analyses (PEAKS-X) of a series of four limited trypsin proteolysis (15, 20, 25 and 30 min) LC-MS sample measurements.

Results

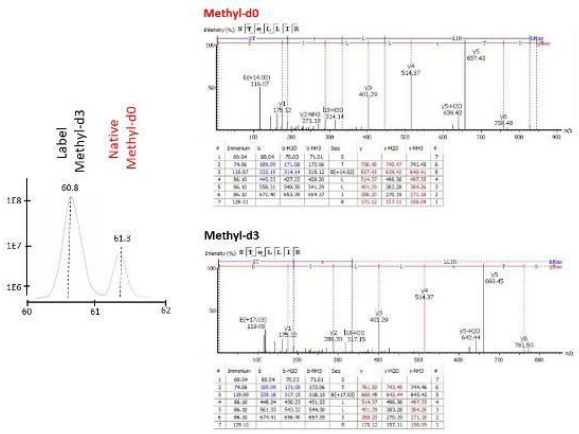
Following manual curation, we detected and classified three types of D/E O-me sites on all four core histones, namely sites (i) exclusively identified with methyl-d3 (+17.03 amu), (ii) exclusively bearing methyl (+14.02 amu), or (iii) harboring both types. Reactivity for methanol-d4 might depend on accessibility (residual protein structure), the sequence context and presence of other PTMs. Non-labeled O-me groups in H2A were exclusively detected in the acidic patch. Likewise, they also cluster in the H2B acidic patch, although some are mapped to other parts of H2B. In H4, all D/E sites can carry light O-me groups, while in H3 this is only the case for sites in the histone core domain. LFQ quantification suggests very low stoichiometry of D/E O-me (0.01-0.05%). Other small nuclear proteins (acidic histone extract background) were either in class (i) or (ii), despite being rich in D/E residues. Notably, proteins introduced post gel-staining were not subjected to methanol-d4 labeling.

Conclusions

Overall, we observed more E than D O-me sites, and D/E O-me seems to be rather specific, e.g. 33% of H2A D/E residues were found to be modified. Since we can virtually rule out sample preparation introduced methylation artefacts, we propose that +14.03 amu O-me sites are endogenous. Until now, D O-me was described to function during cellular protein repair. Since the histone H2A/H2B acidic patch regions are involved in chromatin folding, dimer-tetramer interaction towards H4 and present known binding platforms for nucleosome modifiers (Dann, Nature 2017), D/E O-me might modulate these events. Interestingly, quite some of the newly identified E O-me sites (In H3 and H2B) are among the most prevalent mutated residues present in oncohistones (Bagert, NatureChemBiol 2021).

Poster presentation

Fig. 1



P108

Investigating the role of *Arabidopsis* plastidial HISTONE DEACETYLASE in photosynthesis

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Lysine acetylation is an important post-translational-modification (PTM) that plays a vital role in plant development and the responses to different environmental stimuli. While most work has focussed on histone acetylation the importance of non-histone lysine acetylation has become more and more apparent in recent years. Proteins of many different organelles have been found to be lysine-acetylated including those from chloroplasts. Histone deacetylases (HDACs) are responsible for removing lysine acetylation on various proteins. From the 18 HDACs found in Arabidopsis only HDA14 has been found to localize in plastids. In a previous study performing a mass spectrometry-based lysine acetylome profiling 1,022 *Arabidopsis* protein groups with acetylation sites were identified. 43 % of these sites were associated with the chloroplast and 10 % directly with photosynthesis. This gives great importance to the study of HDA14 and its potential role in regulating photosynthesis. Here we use different co-immunoprecipitation approaches to help identifying possible interaction partners. Many co-immunoprecipitated proteins showed a plastidial localization further strengthening the role of HDA14 as an important plastidial deacetylase.

P109

Proteomic and Phosphoproteomic Analysis of Primary Myeloma Plasma Cells Identifies Distinct Phosphorylation Events Associated with Resistance to Proteasome Inhibitors using an Ex Vivo Drug Sensitivity Testing Platform

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Objectives: Multiple myeloma (MM) is a malignancy of plasma cells in the bone marrow resulting in end organ damage. The five-year survival rate of MM has doubled in the last 20 years owing to the introduction of new treatments such as proteasome inhibitors (PIs), immunomodulatory drugs and more recently monoclonal antibodies and CAR T-cell therapy. MM remains an incurable disease due to persistent relapse and resistance to chemotherapy. This highlights the urgent need for predictive biomarkers of therapeutic response to establish a personalized approach to therapeutic decision-making. This project aimed to perform a phosphoproteomic analysis of primary MM plasma cell lysates stratified based on their ex vivo drug response profiles to the PIs, Bortezomib and Carfilzomib, to improve our understanding and identify novel biomarkers of chemoresistance.

Methods: $CD138^{+}$ plasma cells were isolated from 20 adult MM patient bone marrow aspirates at diagnosis or relapse. Peptides were generated and purified by filter aided sample preparation. Peptide tandem mass tag (TMT) labelling, phosphopeptide enrichment using immobilized metal ion affinity chromatography, synchronous precursor selection, and triple stage tandem mass spectrometry was performed. Nonenriched peptides were used for proteomic analysis. Patients were stratified into groups of "most sensitive" (n=6) and "least sensitive" (n=6) to two proteasome inhibitors, Bortezomib and Carfilzomib, based on ex vivo drug sensitivity scores (DSS) as described previously [1, 2]. Data was analysed using MaxQuant, Perseus and G:profiler.

Results: Almost 3,000 phosphorylation sites from 690 phosphoproteins were identified. Of these, 88 and 47 phosphosites were significantly changed in MM patients "most sensitive" and "least sensitive" to

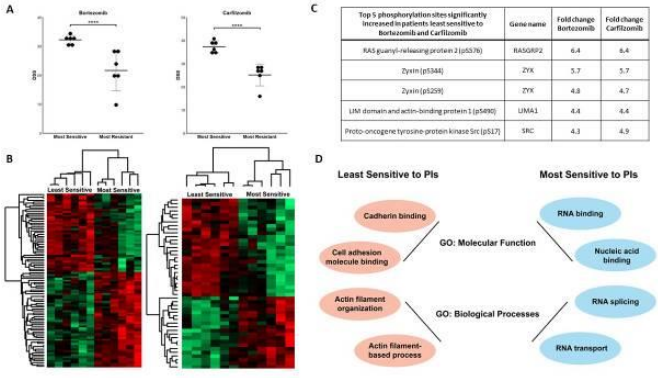
Bortezomib and Carfilzomib, respectively (FDR < 0.05). Distinct phosphoproteomic profiles associated with the two patient subgroups were identified. Proteomic analysis identified upregulated proteins previously reported to be associated with drug resistance in MM indicating the reliability of this analysis for the detection of markers of therapeutic response. Gene ontology (GO) analysis of significantly increased phosphoproteins in "least sensitive" samples demonstrated an increased phosphorylation of proteins associated with cell adhesion and actin filament-based processes. Phosphoproteins increased in "most sensitive" patients revealed an increased phosphorylation of proteins involved in RNA binding and splicing.

Conclusion: The detection of protein abundance and phosphorylation site occupancy provides an in-depth characterization of the molecular mechanisms of chemoresistance/sensitivity in MM. Candidate markers of therapeutic response to PIs were identified and further analysis of these phosphorylation sites should aid the development of novel therapeutic approaches.

[1] M. Majumder et al., Oncotarget 8(34), 56338 (2017)

[2] C. Tierney et al., Sci Rep 11, 12866 (2021)

Fig. 1



Supplementary Figure: A. Dot plots with error bars illustrating the drug sensitivity scores (DSS) of the 'most sensitive' and 'least sensitive' MM patient samples to Bortezomib (left) and Carfilzomib (right). **B.** Hierarchical clustering analysis of significantly dysregulated phosphorylation sites in 'least sensitive' and 'most sensitive' MM patient samples to Bortezomib (left) and Carfilzomib (right). **C.** List of the top 5 phosphorylation sites significantly increased in patients 'least sensitive' to Bortezomib and Carfilzomib. **D.** Gene ontology (GO) analysis of phosphoproteins found to be significantly increased in 'least sensitive' samples and significantly increased in 'most sensitive' samples.

P111

Development of a mass-spectrometry-compatible chemical probe for the enrichment of protein citrullination

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Objective

Citrullination is a post-translational modification (PTM) on Arginine catalysed by peptidylarginine deiminase (PAD) enzymes. This modification results in a mass increase of 0.98 Da and the loss of a positive charge affects the tertiary structure of a protein and its function. It has been reported that citrullinated proteins may have a role in neurological diseases such as multiple sclerosis or Alzheimer"s disease and anti-citrullinated protein antibodies are already being used in the diagnosis of rheumatoid arthritis, but the proteomic-wide studies around this PTM are very scarce. The main challenges in the analysis of this PTM are its low stoichiometry and the ambiguous identification by database search due to the same mass change as deamidation of asparagine or glutamine. To overcome these challenges, this project aims to develop biochemical tools for the enrichment of protein citrullination and to explore the citrullination proteomes in body fluids of patients with neuro-inflammatory conditions. This way, we can broaden our knowledge about this PTM and investigate its impact on diseases.

Methods

The reaction of the ureido group of citrulline with glyoxals has been previously described [1] and used in this study. First, synthetic citrullinated peptides were derivatized with a glyoxal derivative followed by a consequent click reaction to a biotin residue. The derivatized peptides can be selectively enriched using streptavidin beads. The products were cleaved to remove the tag before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We then examined the different acquisition methods to investigate the property of the derivatized citrullinated peptides.

Results

The derivatization strategy has preliminarily been performed over a pool of 200 synthetic peptides. In addition, different fragmentation types and acquisition methods have been examined. The spectra were manually inspected for potential diagnostic ions. Next, the information from the manual inspection was added to search engines to evaluate the accuracy and coverage of the identification by using a large-scale database search on cell lysate with spiked synthetic citrullinated peptide.

Conclusion

We have investigated the spectral characteristics of citrullination-derivatives and integrated this strategy in the large-scale database search. Further evaluation on other sample types (e.g. tissue and body fluid) and establishment of an automated pipeline will be followed.

[1] Tutturen, A. E.; Holm, A.; Fleckenstein, B. Specific biotinylation and sensitive enrichment of citrullinated peptides. Anal. Bioanal. Chem. 2013, 405 (29), 9321−31.

P112

Quantitative phosphoproteomics identifies filamin C as a target of protein phosphatase 1 in skeletal muscle cells during acute mechanical stress

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Objective: In exercising skeletal muscle cells, mechanical strain activates mechanical sensors such as filamin C (FLNc) and integrins, which elicit signaling pathways governed by protein kinases and phosphatases to control and maintain contractile functions. Changes in protein phosphorylation pattern regulate key players and their interactions within the cellular proteostasis network, which enable cells to adapt and protect themselves from damage in consequence of mechanical stress. In this work, we designed a functional phosphoproteomics approach to identify regulatory mechanisms involved in mechanical stress protection in skeletal muscle cells.

Methods: To delineate the mechanical stress-activated signaling network in C2 myotubes, we performed global quantitative phosphoproteomics using triple stable isotope dimethyl labeling in combination with high pH RPLC for peptide fractionation and IMAC for phosphopeptide enrichment. Phosphoproteomics data mining was performed using computational methods to reveal stress-activated signaling pathways and key targets of the proteostasis network. To further study the FLNc interactome in C2 cells, quantitative proximity proteomics and pulldown assays were performed. Functional proteomics and biochemical analyses were performed to identify kinases and the protein phosphatase targeting FLNc to modulate its protein interaction(s).

Results: We established a vast phosphoproteome with 33,000 phosphosites localized in 7,226 proteins of skeletal myotubes. 21,428 phosphosites were quantified in ≥ 3/6 biological replicates. Under acute mechanical stress, 2,243 phosphopeptides were significantly up- and 700 downregulated. Kinase-substrate enrichment analysis showed that mechanical stress markedly increased the activity of JNK1, p38α, ERK1 and PKCα. We identified the signaling adaptor FLNc and several proteins of the associated chaperone-assisted selective autophagy (CASA) machinery as a hotspot of (de)phosphorylation events. Filamin A-interacting protein 1 (FILIP1) was confirmed as a novel direct interacting partner of FLNc, whose binding is regulated by dual-site phosphorylation at S2234/S2237. AKT- and PKCα-mediated phosphorylation of these sites reduces the binding affinity to FILIP1 and thereby protects FLNc from FILIP1-mediated degradation. Furthermore, we identified the protein phosphatase 1 (PP1) to specifically bind to FLNc in the same region. Using phosphatase inhibitors and activators, we confirmed that PP1 dephosphorylates FLNc at these sites both *in vitro* and in cells. We propose that PP1-mediated dephosphorylation of FLNc leads to increased binding

of FILIP1 to mediate the removal of damaged FLNc in mechanically stressed skeletal muscle cells.

Conclusion: Quantitative phosphoproteomics enabled to identify the PP1-dependent dephosphorylation of FLNc, which provides a mechanism for FILIP-mediated removal of less- or non-functional FLNc under acute mechanical stress.

P113

Proteolytic remodelling in mouse model of polycystic kidney disease (PKD) elucidated by bioinformatic extraction of truncated peptides from LC-MS/MS

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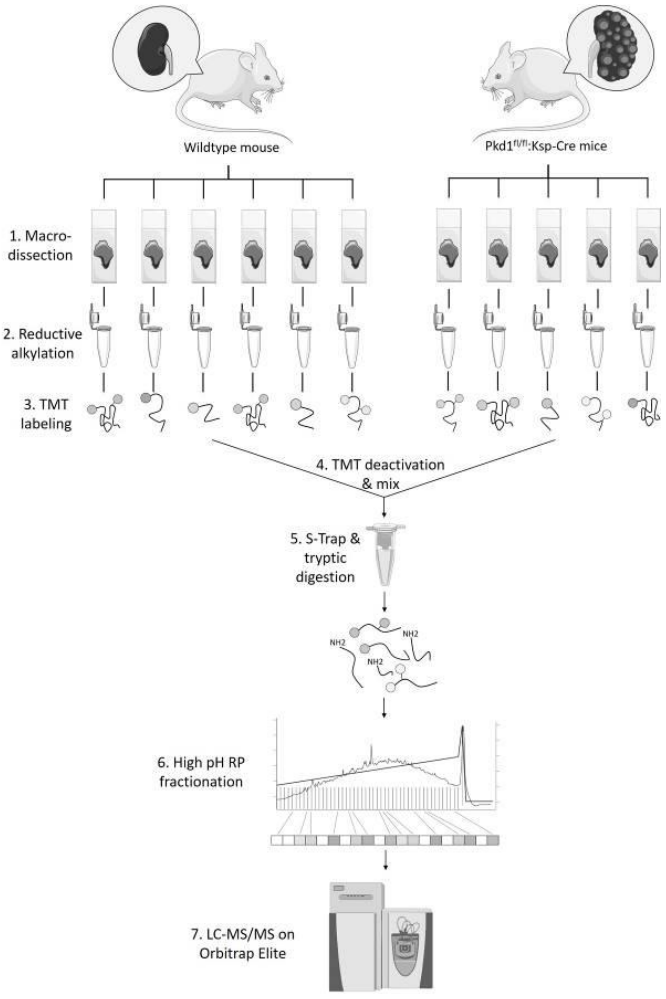
Dysregulated expression and activity of proteases has been linked to the development of various diseases, including polycystic kidney disease, cancer, blood clotting disorder or Alzheimer. This requires for a comprehensive characterization of the proteome in order to gain insights into its functional state, including proteolytic events associated with pathological processes or regulatory mechanisms. A common readout of protease-based mechanisms is based on the quantitative analysis of protein N-termini using bottom-up liquid chromatography – tandem mass spectrometry (LC-MS/MS). In this context, this study focused on optimizing the procedure of N-Terminal Amine Isotopic Labeling of Substrates (NTAILS) which was initially developed by Kleifeld et al. (2010). The aim is to achieve higher sensitivity, better proteome coverage as well as higher throughput than the initial protocol. The proposed strategy is based on one-pot-chemistry and an improved negative selection of N-terminal peptides using fluorine-based reagents instead of high molecular weight polymers. For the initial experiments, a synthetic quality control peptide and HEK293T cells were utilized. Moreover, standard N-terminomic workflow for a robust and proteome-wide identification of both endogenous N-termini of proteins and endogenous neo N-termini of truncated protease substrates, as well as protease cleavage specificity was optimized. Therefore, isobaric labeling of proteins and extensive fractionation via high pH reversed phase chromatography were employed. Data acquired in this study was analyzed using liquid chromatography and tandem mass spectrometry (LC-MS/MS) on Orbitrap Elite and both exploratory and inferential analysis were performed in combination with modern search algorithms and in-house R scripts. Applying this workflow on a murine cohort of polycystic kidney disease resulted in identification of 27 495 peptides. Furthermore, a semi-tryptic analysis enabled assessment of 3 423 semispecific N-terminal peptides. This approach focuses on identification of low-abundant proteoforms, non-canonical protease cleavage sites and also potential biomarkers in a systematic and reproducible manner.

Fig. 1



Poster presentation

P114

Efficient mass spectrometric identification of N-glycopeptides: Improvement from collision energy optimization

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Objective: Glycosylation is one of the most common post-translational modifications in which diverse glycan structures are connected to proteins. The resulting glycoproteins play key roles in various biological processes. Mass spectrometry coupled to liquid chromatography is an indispensable tool in their analysis due to their wide variety and low concentration. Tandem mass spectrometric experiments are evaluated by database search, based on comparison with theoretical spectra. Besides the characteristics of the software used, the success of the identification is influenced by the experimental parameters. Setting the collision energy such that the results provide information on both the glycan and amino acid sequence is still not straightforward. Therefore, the present study focused on enhancing the efficiency and reliability of mass spectrometric measurement and evaluation methods suitable for the identification of N-glycoproteins.

Methods: Hundreds of nano-LC-MS/MS measurements on tryptic digest of standard glycoproteins (alpha-1-acid glycoprotein, fetuin and transferrin) were taken by systematically changing the collision energy, including both single energy and stepped energy methods. Data analysis was performed using various search engines (Byonic, pGlyco, GlycoQuest). Energy dependence of identification score for several hundred N-glycopeptides was fitted using Gaussian functions. Based on the results on the individual glycopeptides, we designed an optimal mass spectrometric measurement strategy which was tested on various complex samples.

Results: The optimal energy depends on the applied software and, in specific cases, on the structure of the glycan, thus offering the possibility to fine-tune the collision energy setting. In performance tests on glycopeptide enriched HeLa, human plasma and glycopeptide enriched human plasma samples, 17-189% more glycopeptides with 3-58% higher reliability score on average were identified with the optimized method as compared to the literature approach depending on the sample and on the applied search engine. Further tests on lung tissues sections for cancer biomarker study also showed significant improvement.

Moreover, a handful of N-glycopeptides was chosen as reference list and a protocol was proposed for methodology transfer to other instruments.

The optimal collision energy dependence on structural features corroborated the peptide centric scoring algorithm of Byonic software. Strong dependence on charge was found for GlycoQuest and glycan score of pGlyco. Finally, the number of labile sialic acid groups inversely correlated with the optimal collision energy for almost all cases. Opposite trend was obtained for Byonic scores.

Conclusion: We pointed out the importance of collision energy fine tuning. Moreover, we established a protocol based on the measurement of reference glycopeptides as a starting point for faster optimization of collision energy for other types of mass spectrometers.

Fig. 1

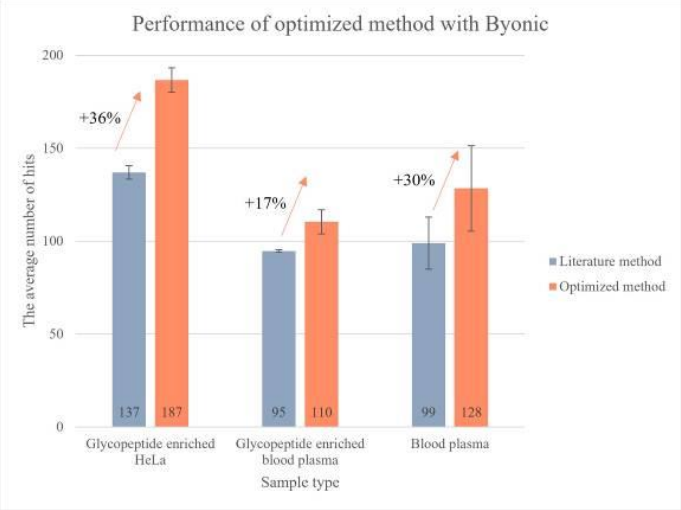
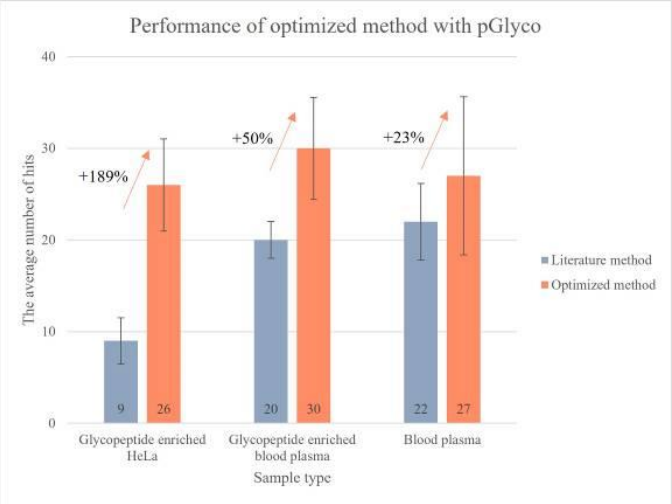


Fig. 2



P115

A robust method for chemical release and analysis of protein-bound N-glycans via horizontal fluorescence assisted carbohydrate electrophoresis in a HPE™ Blue Horizon™ cooled flatbed chamber

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Protein bound N-glycans are a major constituent of manufactured therapeutic glycoproteins. Due to variations in host cell performance during upstream processing at production scale the resulting glycoprotein therapeutics are characterized by N-glycan moieties composed of varying numbers of glycosidically linked hexose and deoxyhexose units. This heterogenic profile of N-glycan compositions within a manufactured batch of bulk drug substance affects the overall efficacy of the biologic drug and is therefore considered to be a critical quality attribute that needs to be monitored throughout production and prior to product release. Process analytical technology for assessing N-glycan composition in an industrial context not only requires affordable costs per measure point but also rugged equipment and rapid, robust, easy to implement methods that ensure reliable long-term performance of the glycan analysis system in a process critical setting.

Here we describe an analytical solution that facilitates the time- and cost-efficient processing of multiple glycan samples in a single run. This newly developed chemical glycan release procedure is based on a system of stable reactants that are able to generate and recycle the highly reactive electrophilic glycan release agent in-situ. C18- reversed phase and HILIC-HPLC-FLD analysis revealed the N-glycan profiles of the chemically released N-glycans to be consistent with those obtained by standard PNGase F treatment. Time and cost reduction in sample preparation and analysis is achieved by elimination of an otherwise essential sample cleanup step combined with the direct analysis of multiple glycan preparations (n=24) in a single separation run. The direct analysis of fluorescently labelled glycans on a horizontal gel electrophoresis system with lot controlled precast gels and validated methodology allows for consistent and accurate characterization and quantification of large sets of incurred samples from biomanufacturing runs and circumvents the need for laborious sample clean-up.

P116

A method platform to make intact-glycoproteomics more accessible

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Objective: Analyzing the glycoproteome is challenging, as glycosylation is a non-templated PTM and varies on several levels. Glycoproteins have several glycosites, each of which might be occupied at a given time (macrohetrogeneity) with a glycan that can also vary in structure (microheterogeneity). Despite this, changes in the glycoproteome hold a wealth of biological information, that is inaccessible *via* standard/quantitative proteomics. Currently the barrier to incorporate glycoproteomic data in biological evaluations is still high, due to complexity, inaccessibility and time expenditure. This becomes even more pronounced when the objective is to analyze intact glycopeptides (i.e. glycan and peptide IDs from a single experiment). In addition, there is currently no "*best practice*" protocol available – hence we believe that a limited pool of methods to facilitate analysis of glycoproteome sub sets is the best solution for now.

Methods: We currently develop a modular method platform that can be used to facilitate experiment from

a first "fingerprinting" to a detailed analysis of the glycosylation status of complex biological systems. We aim to establish a set of "*pick and choose*" standard protocols (depending on the study-aim) for the crucial steps of sample preparation: lysis, digest, enrichment and MS-analysis.One major aspect of this platform will be the application of chemo-proteomic solutions via the use of modular, bioorthogonal probes. We plan to combine multi-functional, cleavable probes and isotope labeling to facilitate enrichment and subsequent quantitation. Basis for this is the well-established incorporation of non-natural sugar moieties via the metabolic pathway. A first-generation library consists of probes reliant on either azide- or alkyne- groups as the biorthogonal handle, an isotope labelled core connected *via* various cleavable linkers, to various readouts (e.g. Rhodamine B for imaging) and functional handles (biotin for enrichment, photo-crosslinker, etc.)

Results:The prototype platform relies on versatility, robustness, ease of use and time efficiency. The current workflow takes approx. 6.5 hours to prepare e.g. 6 samples for analysis, and allows for a fingerprinting that captures even facile differences in N-glycosylation (on the level of both micro- and macrohetrogeneity). Using models for either cancer or congenital disorders of glycosylation (CDGs), we mapped several hundred intact glycopeptides on proteins spanning from ER-resident (high mannose glycans) to cell-surface (branched / complex glycans) proteins.Conclusion:Combined with current advances and availability of relevant data analysis tools, we believe that this method platform can help to lower the hindrance to include glycoproteomic analysis in biological projects thereby giving access to this often-unexplored information. At the same time, we show that even at the non-expert level, meaningful data can be generated and built upon for further studies.

P117

Characterization of complex glycosylation patterns using a new QTOF mass spectrometer with EAD

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Fetuin is a frequently used MS/MS test molecule with a complex N--and Oand O--glycosylation profile. Comprehensive glycosylation profile. Comprehensive characterisation of the Ocharacterisation of the O--linked glycans is particularly challenging due to the proximity of four of the five linked glycans is particularly challenging due to the proximity of four of the five previously reported Opreviously reported O--glycosylation sites (S271, T280, S282, S296), which in combination with the relative glycosylation sites (S271, T280, S282, S296), which in combination with the relative cleavagecleavage--sites of standard digestion enzymes, results in a single tryptic peptide of 61 amino acids containing sites of standard digestion enzymes, results in a single tryptic peptide of 61 amino acids containing four possible Ofour possible O--glycosylations [1,2].glycosylations [1,2]. Electron associated fragmentation is particularly suitable for glycopeptide sequencing because it induces strong Electron associated fragmentation is particularly suitable for glycopeptide sequencing because it induces strong cleavage of the peptide backbone, in preference to glycan fragmentation as is commonly observed with cleavage of the peptide backbone, in preference to glycan fragmentation as is commonly observed with CID/HCD [3]. Here we present results acquired using electron activated dissociation (EAD), a form of hot CID/HCD [3]. Here we present results acquired using electron activated dissociation (EAD), a form of hot electron capture dissociation, available on a newly developed SCIEX QTOF instrument called the ZenoTOF electron capture dissociation, available on a newly developed SCIEX QTOF instrument called the ZenoTOF 7600 system.7600 system.

The combination of the ZenoTOFZenoTOF7600 system and the Biologics Explorer software can perform comprehensive 7600 system and the Biologics Explorer software can perform comprehensive characterisation of biologics. The results for the particularly challenging case of fetuin characterisation of biologics. The results for the particularly challenging case of fetuin OO--linked glycans linked glycans demonstrate this capability.demonstrate this capability. In this work the algorithm used to score a putative sequence against an MS/MS spectrum did not consider In this work the algorithm used to score a putative sequence against an MS/MS spectrum did not consider actual glycan fragments themselves. As mentioned, EAD fragmentation predominantly induces cleavage of the actual glycan fragments themselves. As mentioned, EAD fragmentation predominantly induces cleavage of the peptide backbone rather than the glycan, so this was not a significant limitation. Nonetheless future work may peptide backbone rather than the glycan, so this was not a significant limitation. Nonetheless future

work may also consider these fragments.also consider these fragments.

P118

Analysis of urinary O-glycopeptides: Systematic evaluation of a dataset using different search engines

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Objective

Search engines can confidently identify unmodified peptides from proteolytic digests. However, the reliability of identifications related to modified peptides including O-glycopeptides is much lower. O-glycopeptides are made up of two types of compounds that exhibit different fragmentation characteristics. During data analysis not just the peptide but also the modifying glycan(s) have to be identified. As O-glycopeptide identification is a more complex task than the identification of unmodified peptides the aim of the study was to see that what kind of information search engines consider from spectra that were recorded with different activation techniques (HCD and EThcD).

Methods

O-glycopeptides were enriched from tryptic digests of ten donors" urine using a two-round wheat germ agglutinin lectin affinity chromatography. The enriched samples were then subjected to LC-MS/MS analysis. For all selected precursors an HCD spectrum was recorded. If an HCD spectrum contained the HexNAc oxonium ion (m/z 204.0867 ± 10 ppm) among the 20 most intense ions then an EThcD spectrum was also recorded. Three database search engines (Byonic, Protein Prospector and Meta Morpheus) and one utility program (Protein Prospectors" MS-Filter) were used in the evaluation process to see how similarly/differently they work.

Results

All search engines identified more than a hundred O-glycosylated urinary proteins. From the data it is obvious that in general all search engines can reliably identify the peptide part. In some instances, the search engines delivered different peptide sequences with the same additive glycan mass. Only Meta Morpheus delivered the right peptide sequence despite the fact that there was not enough peptide fragment in the HCD spectrum. This was achieved by the utilization of the Y_0 ion in the HCD spectrum and the concerted use of the EThcD spectrum from which the search engine identified the correct peptide sequence. Characterization of the glycan part is more problematic. In general, the search engines handle the O-glycans only as an additive mass and no structural evaluation was made.

Conclusion

EThcD spectra contain far more information than search engines consider during the identification process. Besides the peptide backbone fragments EThcD spectra contain Y and B ions from which structural information can be obtained on the modifying O-glycan(s). For example, with the help of these ions peptides modified with isomeric O-glycans can be distinguished and characterized. Reliable site determination is still the toughest part of O-glycopeptide characterization. This is due to multiple factors the most relevant being is the low fragmentation efficiency of the ETD process. Among the tested search engines, the most suitable for automated O-glycopeptide identification is Meta Morpheus. This is the first search engine that combines information from both HCD and EThcD spectra.

P119

Alterations in protein expression and site-specific N-glycosylation of prostate cancer tissues

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Objective:

Prostate cancer (PCa) is one of the most prevalent types of cancer in males, however, much is still unknown about the molecular and subsequent biological changes during disease progression. Our goal was the detection of alterations in N-glycosylation and additionally protein expression levels between

different pathological grades of PCa and healthy tissue samples.

Methods:
Tissue microarray biopsy samples (TMAs) were analyzed, 95 in total. The protein content of a single TMA
core was approximately 1 µg, which was digested on the tissue surface using trypsin enzyme, then extracted. This was followed by sample clean-up using C 18 solid phase extraction, and glycopeptide enrichment by acetone precipitation. After the enrichment, the glycopeptide-rich pellet fraction and the
supernatant fraction were analyzed separately using a nano-HPLC-MS/MS system. Protein identification and quantitation was performed using MaxQuant, on the other hand for glycopeptides the combination of Byonic and GlycoPattern was used. Data analysis was carried out in STRING, Perseus, and RStudio using R.

Results:
Among the 95 samples analyzed altogether 653 proteins and 145 N-glycopeptides were quantified. Out of these proteins 123 were found to be differentially expressed between healthy and PCa tissues, 72 up- and 51 downregulated, corresponding to metabolic, and cellular component organization processes respectively. Multiple glycosylation features were also found to be altered between these two groups, including the abundance of 7 glycopeptides, and the sialylation, fucosylation and galactosylation on 9 glycosites.
Significant molecular changes were also identified with disease progression, between the different grades of PCa and healthy tissue. This included the altered expression of 75 proteins, the abundance of 4 glycopeptides, and the altered fucosylation at 1 glycosite.
The glycoproteins identified with altered glycosylation were all secreted to either blood or the extracellular matrix, and according to the Human Protein Atlas most of them are unfavorable prognostic cancer markers. Our results also show, that changes in glycosylation primarily occur at the glycosite level while overall glycosylation remains constant.

Conclusion:
Protein expression levels and site-specific N-glycosylation of 95 healthy and cancerous tissue samples
were determined, and the corresponding biological processes identified. Many of these molecular differences are potential diagnostic, and prognostic marker candidates, however, their cancer specificity needs further confirmation.

Conflict of interest:
The authors declare no conflict of interest.

Acknowledgements:
Funding by the National Research, Development, and Innovation Office (PD 121187 and FK 131603) and support of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences is acknowledged.

P120
Obtaining of recombinant protein C activator (*Aspergillus ochraceus* protease) with potentially usage in medicine

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The *Aspergillus ochraceus* VKM-F4104D micromycete is a producer of extracellular protease with activating human plasma protein C activity. Protein C strongly inhibits thrombin formation, stimulates fibrinolysis and plays the role of a cytoprotective signal molecule. The lack of protein C leads to different cardiovascular complications and, thus, the determination of it is required for the diagnostics and therapy of cardiovascular diseases.

Protease activating protein C (PAPC-4104) of *A. ochraceus* VKM-F4104D shows similar properties with exogenic protein C activator from snake venom and can be applied in practical medicine.

PAPC of micromycete *A. ochraceus* VKM-F4104D was obtained in recombinant form using a vector pET-23d and *E. coli* BL21 strain. The isolation of recombinant protease was performed using Ni-NTA chromatography. This enzyme, which belongs to the proteinase-K-like proteases, is similar (more than 74%)

to the proteases encoded in the genomes of *A. fumigatus* ATCC MYA-4609, *A. oryzae* ATCC 42149 and *A. flavus* 28. The analysis of *A. ochraceus* VKM-F4104D gene encoding PAPC-4104 showed that mature PAPC-4104 is 282 amino acids long, preceded by the 101-amino acid propeptide necessary for proper folding and maturation. The predicted structure of the peptidase domain revealed the catalytic triad to be composed of Ser-350, His-194, and Asp-41 amino acid residues. Bioinformatic analysis of the gene and mRNA sequences became a base to design a strategy to obtain PAPC-4104 in a functional and soluble form in the simple *E. coli* expression system. Due to primary biochemical property studies, the recombinant protease was identical to the native enzyme from *A. ochraceus* VKM-F4104D in terms of its biological properties, including an ability to hydrolyse chromogenic peptide substrates of activated protein C (pGlu-Pro-Arg-pNA) and factor Xa (Z-D-Arg-Gly-Arg-pNA) in conjugant reactions with human blood plasma. The native and recombinant PAPC had similar molecular weight and demonstrated similar electrophoretic mobility.

Therefore, recombinant PAPC-4104 can potentially be used in medicine, veterinary science, diagnostics, and other applications.

This research was supported by the Russian Science Foundation (grant #20-16-00085).

P121

Proteomics evaluation of semen of healthy Beagle-breed dogs

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Objective. The work refers to a proteomics investigation of semen of healthy dogs, to increase available knowledge in the topic and present reference work for proteomics of canine semen.

Methods. Five adult pure-breed Beagle male dogs were included in the study. Clinical and ultrasonographic examination of the genital system was used to confirm their health. Semen samples were collected. A standard seminological examination was performed and samples were separated in three fractions: pre-sperm, sperm-rich, prostatic. Blood samples were also taken for plasma preparation. Proteomics analysis was applied to sperm-rich and prostatic fractions and to plasma samples, by using 1-D nanoLC-MS/MS analysis (LTQ Orbitrap Elite). All proteins identified were classified based on their functional annotations by using Gene Ontology (GO) for molecular function, biological process and subcellular localization.

Results. All seminological parameters of samples from all dogs were within the normal range. In total, 43 and 38 proteins were identified in prostatic and sperm-rich fraction, respectively, of semen. Also, 59 proteins were identified in plasma.Three proteins were identified concurrently in sperm-rich and prostatic fractions (apolipoprotein E; keratin, type II cytoskeletal 1; nitric oxide synthase, inducible). Two proteins were identified in sperm-rich fraction and plasma (abnormal spindle-like microcephaly-associated protein; nephrocystin-1). Eleven proteins were identified in prostatic fraction and plasma (actin, cytoplasmic 1; arginine esterase; clusterin; coiled-coil domain-containing protein 39; DNA-dependent protein kinase catalytic subunit; glyceraldehyde-3-phosphate dehydrogenase; G2/mitotic-specific cyclin-B3; heat shock 70 kDa protein 1; serum paraoxonase/arylesterase 2; tight junction protein ZO-3; ubiquitin-60S ribosomal protein L40). In sperm-rich fraction, most proteins were related to cell cycle (n=15) or cell organization-biogenesis (n=6); in prostatic fraction, most proteins were related to cell organization-biogenesis (n=19), metabolic processes (n=6) or ion/molecule transport (n=6). In sperm-rich and prostatic fractions, most proteins were associated with cell membrane (n=15 and 18, respectively), cytosol (n=15 and 13) or nucleus (n=13 and 10).

Conclusions. The results provide reference values for proteins in semen of dogs, for use in future comparisons and experiments. Differences were seen in biological processes in which predominating proteins in prostatic and sperm-rich fractions, were involved, but not in their subcellular localization. Further studies should evaluate semen samples from animals with genital disorders, for example, prostate neoplasia or genital system infection (e.g., *Brucella canis* infection), which will be useful to clarify the pathogenesis of these disorders and to identify biomarkers for their diagnosis.

P122

Using entomopatogenic nematodes and entomopatogenic fungi as biocontrol agents against to date Palm tree borers, *Oryctes* spp. (Coleoptera: Scarabaidae: Dynastinae)

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The efficacies of the entomopatogenic nematodes (EPN), ***Metarhabdits blumi***, and the entomopatogenic fungi (EPF), ***Beauveria bassiana*** as a biocontrol agents against were determind date palm tree borers, ***Oryctes*** spp. (Coleoptera: Scarabaidae: Dynastinae) in laboratory and field trails, during 2018 season. Laboratory results demonstrated that direct spray of 1000 infective juveniles (IJs) per ml of ***M. blumi*** on Arabian Rhinoceros Beetle, ***Oryctes agamemnon arabicus*** (ARB) larvae caused 71.67% and 15% mortality in the adults. While, treating the food source of the larvae (pieces of fresh tissue of the frond bases) with the same dose and period resulted in 48.33% mortality in larvae and 10% in the adults. Laboratory results also showed that using concentration 1×109 conidia/ml-1 of ***B. bassiana*** as direct spray of the ARB larvae, led to 66.7% and 60% as treatment of the food source. Field experiments results showed that injection of 50 ml per palm tree with a concentration of 1000 IJs/ml of ***M. blumi*** inflected about 42% mortality in ARB larvae infested the tree. Meanwhile, injection 50 ml of 1×109 conidia/ml-1 of ***B. bassiana*** imposed 50% mortality in larvae. Results of this investigation illustrate the possibility of using ***M. blumi*** and, ***B. bassiana*** as a bicontrol agents against palm borers in IPM programs.

Kerwords: Entomopatogenic nematode, ***Metarhabdits blumi,*** Entomopathogenic fungi, ***Beauveria bassiana ,*** biocontrol, endophyta, Palm borers, ***Oryctes*** spp.

P123

Plasma biochemistry and proteome modulation of loggerheads during recovery

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The aim of the study was to monitor and analyse loggerhead sea turtles (*Caretta caretta*) plasma proteome profiles and biochemistry parameters during their recovery period in rescue centre within different age and recovery period groups, and determine the potential biomarkers that can be used in diagnostics.

The biochemical parameters were determined by use of clinical chemistry analyser DiaSys respons®910 (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). High-resolution liquid chromatography tandem mass spectrometry analysis of loggerheads plasma was carried out on UltiMate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The proteome analysis of plasma samples was performed by using shotgun TMT-based quantitative approach using TMTsixplex Isobaric Label Reagent Set (Thermo Scientific Pierce Biotechnology, Rockford, IL, USA).

The plasma biochemical parameters of total protein and glucose content, accompanied by aspartate aminotransferase and N-acetyl-cystein-activated creatinine kinase were the most frequently modulated biochemical parameters in injured loggerheads during recovery. Using high-throughput TMT-based proteomic approach in plasma samples upon arrival of injured, and before release of recovered sea turtles to natural environment, we identified 29 significantly differentially abundant proteins in loggerheads age and recovery period groups (Fig. 1). Out of 17 REATCOME pathways, those proteins were mostly recognized and included in: *(i)* post-translational protein phosphorylation, *(ii)* regulation of Insulin-like Growth Factor transport and uptake by Insulin-like Growth Factor Binding Proteins, *(iii)* innate immune system, *(iv)* platelet degranulation pathways, and *(v)* regulation of complement cascade (Table 1). The alpha-fetoprotein was the only protein which showed statistically

significant up-regulation patterns in all loggerhead age groups before release from the rescue centre, and the complement component 3 protein was the only protein modulated in all recovery period groups. Furthermore, complement component 3 protein takes part in 9; and followed up with apolipoprotein A-I in 7; complement component 4, complement component 5, and kininogen-1 in 6 REATCOME pathways.

The loggerheads plasma proteome profiles and highlighted biochemical parameters in loggerhead sea turtles during rehabilitation were shown to be considered as valuable indicators of turtle's health status and condition. Thereby, the particular pathways and several differentially abundant proteins are highlighted and recommended as potential biomarkers of turtle's health status and condition.

Fig. 1 - Protein abundance levels in loggerheads age groups and recovery stay period groups before turtles release into environment

Table 1 - Modulated proteins and corresponding REACTOME pathways identified in plasma samples of loggerheads recovery period and age groups

Fig. 1

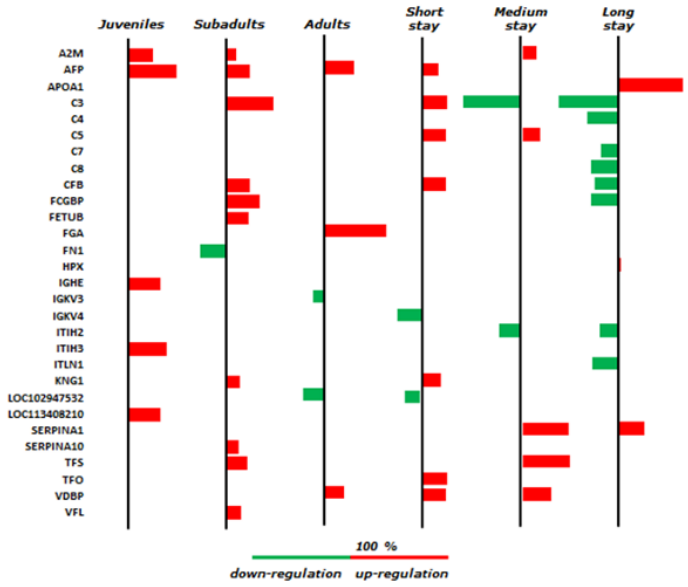

Fig. 2

Term ID	Term description	recovery period groups		age groups	
		FDR	proteins	FDR	proteins
HSA-8957275	Post-translational protein phosphorylation	1.72×10^{-12}	AFP, APOA1, C3, C4, ITIH2, KNG1, SERPINA1, TFS	6.02×10^{-9}	AFP, C3, FGA, KNG1, SERPINA10, TFS
HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	2.69×10^{-12}	AFP, APOA1, C3, C4, ITIH2, KNG1, SERPINA1, TFS	7.11×10^{-9}	AFP, C3, FGA, KNG1, SERPINA10, TFS
HSA-977606	Regulation of Complement cascade	8.52×10^{-11}	C3, C4, C5, C7, C8, CFB	2.90×10^{-5}	C3, CFB
HSA-174577	Activation of C3 and C5	1.84×10^{-9}	C5, C3, C4, CFB	1.40×10^{-4}	C3, CFB
HSA-114608	Platelet degranulation	8.31×10^{-7}	A2M, APOA1, KNG1, SERPINA1, TFS	$5.17E\times10^{-5}$	A2M, FGA, KNG1, ITIH3, TFS
HSA-166665	Terminal pathway of complement	8.61×10^{-7}	C5, C7, C8		
HSA-168249	Innate Immune System	6.73×10^{-6}	C3, C4, C5, C7, C8, CFB, ITLN1, SERPINA1		
HSA-166663	Initial triggering of complement	7.83×10^{-6}	C3, C4, CFB		
HSA-173736	Alternative complement activation	8.23×10^{-5}	C3, CFB		
HSA-8963896	HDL assembly	1.30×10^{-4}	A2M, APOA1		
HSA-2168880	Scavenging of heme from plasma	3.60×10^{-4}	APOA1, HPX		
HSA-140837	Intrinsic Pathway of Fibrin Clot Formation	7.00×10^{-4}	A2M, KNG1	8.10×10^{-4}	A2M, KNG1
HSA-418594	G alpha (i) signalling events	1.00×10^{-3}	APOA1, C3, C5, KNG1		
HSA-375276	Peptide ligand-binding receptors	1.70×10^{-3}	C3, C5, KNG1	3.19×10^{-2}	C3, KNG1
HSA-5653656	Vesicle-mediated transport	5.70×10^{-3}	APOA1, HPX, SERPINA1		
HSA-140877	Formation of Fibrin Clot (Clotting Cascade)			3.72×10^{-3}	A2M, FGA, KNG1
HSA-173736	Alternative complement activation			9.67×10^{-5}	C3, CFB

P125

Characterization of extracellular vesicle cargo in equine osteoarthritis through a SWATH-MS proteomics approach

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Objective: Extracellular vesicles (EVs) carry bioactive molecules, including proteins for cell communication. EVs from one cell type can travel to cartilage promoting its regeneration. Thus, their cargo could be used as biomarkers or treatment for osteoarthritis (OA). Here we employed a SWATH-MS approach to characterise the proteomic cargo of plasma and synovial fluid (SF) derived EVs.

Methods: Equine SF was pooled from the metacarpophalangeal and carpal joints from "healthy", OA, and mesenchymal stem cell (MSC) treated joints resulting in a total of 11ml SF (n=25) and treated with hyaluronidase. EVs were isolated using differential ultracentrifugation (dCU); 300g for 10 minutes, 2000g for 10 minutes, 10,000g for 30 minutes and 100,000g for 70 minutes. EVs were also extracted using size exclusion chromatography from 26 equine plasma samples (4 horses, 7 time points over 63 days) that underwent a carpal groove OA model totalled 8ml. EVs were isolated used dCU. All samples were denatured with 6M urea/1M ammonium bicarbonate/0.5% sodium deoxycholate before cysteines were reduced with DTT and capped with iodoacetamide. The samples were digested with trypsin/LysC for 3h at 37oC, the concentration of urea was reduced to 1M, and

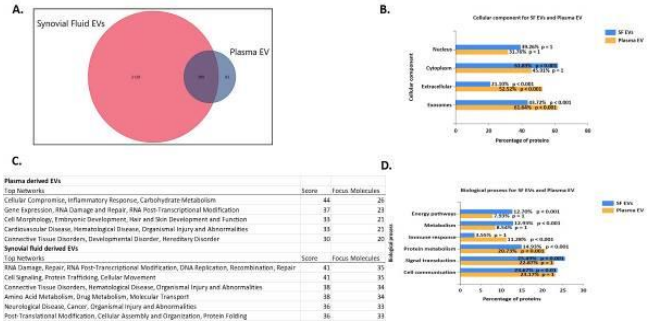
incubation was continued overnight at 37oC. Samples were fractionated on a PolySULFOETHYL A strong cation exchange column and 20 fractions (for each biofluid) were desalted and subjected to nanoLC-MS/MS analysis on a Triple TOF 6600 mass spectrometer. Data were searched using ProteinPilot 5 against the UniProt horse with carbamidomethyl cysteine as a variable modification and biological modifications allowed. Bioinformatics analysis was undertaken using FunRich and IPA.

Results: The plasma library identified 392 proteins and the SF library 2448 protein with FDR 5%. 12.8% of proteins overlapped between biofluids (Fig1A). Based on cellular compartments we show a significant enrichment of the exosome and extracellular component in both biofluids (Fig B). We identified the top canonical pathways as EIF2 signalling (p=3.2E-71) and acute phase response signaling (p=2.2E-41); top upstream regulators as MYC (p=9.8E-76) and TGFβ (p=1.5E-38) for SF and plasma respectively. The top networks for each biofluid are in Fig1C and biological processes in Fig1D.

Conclusion: To our knowledge this is the most comprehensive proteome of EVs derived from SF, highlighting the potential for SWATH-MS in EV cargo proteomics. These results laydown the foundation for two future proteomics studies in our lab. We will characterise the protein cargo of EVs excreted by MSCs (as a treatment for OA) into joint SF in experimentally induced equine OA. We hypothesize that purified MSC-derived EVs could be a novel treatment for equine OA. Additionally, we will determine whether the differential EV proteome in OA can be used as biomarkers to diagnose and stratify disease.

Fig1.Bioinformatic analysis of plasma and SF proteomes

Fig. 1



P126

High-throughput TMT-based proteomics for cow's milk proteome characterization and detection of early protein biomarkers in mastitis

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Objective: The occurrence of bovine mastitis (inflammation of the mammary gland) is considered to be one of the biggest challenges on dairy farms, due to reduction of the animal welfare and milk production and, consequently, economic loss for the farm. Therefore, the diagnosis of mastitis at subclinical stage and early treatment is of the utmost importance. However, the lack of clinical symptoms and unambiguous biomarkers for the diagnosis in early disease stage, prevent further advancements. Thus, the objective of this study was to implement the quantitative tandem mass tag (TMT) approach in combination with statistical and bioinformatics data processing for determination of quantitative changes in milk proteomic profiles during development and progression of mastitis on dairy farms.

Methods: Milk samples (N = 138) were aseptically collected on dairy farms after physical examination and before the milking. Based on the results obtained for somatic cell count (SCC) and bacterial culture (BC) examination, milk samples were classified into experimental groups: healthy positive control (group with normal SCC and positive BC, N = 20), healthy negative control (group with normal SCC and negative BC, N = 20), subclinical mastitis (moderately increased SCC and positive BC, N = 80), clinical mastitis (high SCC and positive BC, N = 18). Samples were processed using TMT label-based quantitative approach. High resolution LC-MS/MS analysis was carried out using the Ultimate 3000 RSLCnano system (Dionex, Germering,

Germany) coupled to Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Protein identification and quantification were performed using Proteome Discoverer software (version 2.3., Thermo Fisher Scientific) with SEQUEST algorithm implemented and database search against *Bos taurus* FASTA files. Statistical and bioinformatic analysis were done using R and Cytoscape.

Results: Altogether, 386 quantifiable proteins were identified. There were 145 proteins with statistically different abundance between the groups. Gene Ontology analysis of proteins with significantly altered abundances showed that milk proteins were involved in biological adhesion, immune system process, biological regulation, interspecies interaction between organism, response to stimulus, signalling and others. Based on the statistical and fold change difference between investigated groups complement component 3, cathelicidin-1, cathelicidin-7, lactoferrin, haptoglobin, and chitinase-3-like protein were identified as potential biomarkers for subclinical mastitis.

Conclusion: Shotgun TMT-based high-resolution proteomic profiling allowed identification of potential milk biomarkers for differentiation of mastitis grade in dairy cows. After validation of selected proteins, these findings might provide a valuable contribution to understanding of mastitis pathophysiology and might be applicable to the development of a new cow-side diagnostic tool.

P127

Understanding the molecular mechanism of DDT toxicity by coupling proteomic analysis with histopathological testing

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Dichlorodiphenyltrichloroethane (DDT), a persistent organochlorine pesticide having adverse biological effects on marine organisms, has been reported to contaminate marine environment. The knowledge on molecular mechanisms of its toxicity on marine organisms is limited. The purpose of current work was to evaluate the toxicity of DDT in the hooded oyster *Saccostrea cucullata.* The proteome response on the exposure to sublethal concentrations of DDT should be investigated in order to explain the molecular mechanism of DDT toxicity. The oysters were exposed to various DDT concentrations from 0 - 2000 µg/L for 96 h. The LC50 (96 h) was afterwards calculated as 891 µg/L. Two sublethal concentrations (10 and 100 µg/L) were used for the investigation of histopathological effects and the proteome response by using 2DE-based proteomic approach. Histopathological results showed that DDT caused the alteration of mantle tissue. These included the induction of mucocytes in the mantle epithelium and the inflammatory effect in the connective tissue which was indicated by enlargement of blood sinus and aggregation of hemocytes within the blood sinus. Proteomic results showed, that amongst approximately 500 protein spots that were detected across 2DE gels, 51 protein spots were differentially expressed ($P < 0.01$; fold change > 1.2). Of these, 29 protein spots were identified by LC-MS/MS. These included 23 up-regulated, 5 down-regulated and 1 fluctuating spots. DDT alters the expression of stress response proteins and cytoskeletal proteins, proteins involved in energy metabolism, calcium homeostasis and other proteins of unknown function. Furthermore, we observed that stress response and cytoskeletal proteins are the central targets of DDT action. Additionally, proteomic results clearly elucidated the molecular response of the histopathological changes, which were driven by the alteration of cytoskeletal proteins. Our results improve the current knowledge of toxicity of DDT to histology and molecular response of oyster proteome. In addition, histopathological changes will be beneficial for the development of an appropriate guideline for health assessment of this species in ecotoxicological context.

P128

Association between plasma PACAP level and protein profile of dairy cows

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Objective

Pituitary adenylate-cyclase-activating polypeptide (PACAP) is a neuropeptide, which takes part in the regulation of several physiological processes and molecular mechanisms, such as reproduction, growth, infant nutrition, thermoregulation, energy status, sympathetic and parasympathetic nervous system. The objective of the present study was to compare plasma protein profiles of cows with high and low plasma PACAP levels.

Methods

Holstein cows were grouped by plasma ELISA assay into 2 groups with 10-fold differences of plasma PACAP levels. Differential proteome-analyses were performed by two dimensional gel electrophoresis (2D-PAGE) followed by tryptic digestion and protein identification by liquid chromatography-mass spectrometry (LC-MS).

Results

As a result altogether 210 individual protein spots were detected, sixteen of them showed significant differences ($p<0.05$) in the expression levels between groups. 10 spots showed higher intensities in the high plasma PACAP level group, while 6 spots were more abundant in the low plasma PACAP level cows. The differentially expressed proteins of plasma were in the volume (V%) range of 0.2-2.0 and their isoelectric point at 5.3-8.0. By function, these play a role in the immune system, lipid metabolism and transport mechanism.

Conclusion

Our results indicate that plasma PACAP concentration is related to lipid metabolism and immune status of cattle as the identified proteins provide further evidence to the immunoregulatory properties and the role in lipolysis and lipogenesis of PACAP. Based on the generated protein-protein interaction networks, it is very likely that the proteome-level changes observed in this study are not the result of the direct significance of PACAP on these proteins. Considering that PACAP is a hormone acting on transmembrane receptors, its functions point beyond direct protein-protein interactions and can have broader effects modulated by the initiated signal transduction pathways. Our study can give useful information on the proteome-wide changes in cow plasma elicited by PACAP and in order to get more details, further studies are needed, such as a comparative proteome analysis on PACAP injected vs. non-injected cows to reveal proteins correlated to elevated PACAP levels.

The publication is supported by the EFOP-3.6.1-16-2016-00022 and EFOP-3.6.3.-VEKOP-16-2017-00008 projects. The projects are co-financed by the European Union and the European Social Fund.

P129

Analysis of proteomic changes during myxomatrous degenerative changes of the mitral valve in dogs

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Objective: Myxomatous mitral valve disease (MMVD) is the most common heart disease and the most frequent cause of congestive heart failure in canine species. The MMVD pathology is almost identical in humans and in dogs, the murmur of mitral valve gurgitation appears years before the clinical onset of heart failure. According to the American College of Veterinary Internal Medicine staging system, dogs with diagnosed MMVD may be asymptomatic with no/mild (stage B1) or more advanced (stage B2) mitral valve regurgitation as well as symptomatic, with clinical signs of heart failure (stage C). The study compared quantitative proteome profiles in serum of healthy and dogs with diagnosed MMVD at different stages. The ultimate goal was identification of potential proteomic biomarkers for early diagnosis of MMVD and detection of progression from stage B1 to B2.

Methods: Serum samples from 50 dogs were collected at the time of initial diagnosis and before any treatment. According to findings on clinical

examination and ultrasound imaging four groups were formed: healthy dogs (N= 12), MMVD stage B1 (N=13), stage B2 (N=12) and stage C (N=13). The samples were processed using shotgun, TMT-based quantitative workflow. Top8 data-dependent acquisition was performed on Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) after peptide separation on Ultimate 3000 RSLCnano system (Dionex, Germering, Germany). Protein identification and relative quantification were performed using Proteome Discoverer software (version 2.3., Thermo Fisher Scientific) with SEQUEST algorithm implemented and database search against *Canis lupus* FASTA files. Data analysis was done using R and PANTHER classification tool.

Results: Altogether, 318 proteins were quantified, 33 proteins had statistically different abundance between four experimental groups. The *post-hoc* analysis identified 15 proteins that had significantly different abundances between control group and MMVD stage B1, approximately half were down regulated (N=8) and half up regulated (N=7) in MMVD stage B1. In diseased dogs (stage B1) transferrin receptor and xaa-Pro dipeptidase proteins, classified by gene ontology as metalloproteases, and gelsolin protein, a non-motor actin binding protein involved in FAS signalling pathway were down regulated in dogs with stage B1 of MMVD. In addition, blood coagulation protein (vitamin K-dependent protein S), glycoproteins (haptoglobin-like, glycoprotein 80 and hemopexin) and several binding proteins (C4b-binding protein alpha chain and retinol-binding protein) were up regulated. In comparison to stage B1, three proteins were down regulated (C-C motif chemokine 14-like, complement C3-like and haptoglobin-like), while complement component C7 isoform X1 was up regulated in stage B2.

Conclusion: The quantitative proteome analysis enabled discovery of potential serum biomarkers for early diagnosis of MMVD (stage B1) and differentiation of MMVD stages B1 and B2.

P130

The faecal proteome of cattle reveals a mix of bovine, plant and microbial proteins

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Objectives Faeces is a complex, heterogeneous, mixture of compounds that includes proteins from diet, host and microbiome. Recent research on bovine faeces has focused on the microbiome but here the aim was to identify not only bacterial proteins in bovine faeces but also proteins from the host and the feed, and to find differentially expressed proteins in faeces from two groups of cattle that were fed differently treated barley-based diets.

Methods Healthy continental crossbreed steers (n=217, predominantly Limousin and Charolais; 506 ± 82 days old, 481.5 ± 37.9 kg) were allocated to two groups after stratification on age and weight. After receiving transition diets, animals were fed a diet (114 ± 10 days) composed of mostly barley, which had been treated with either ammonia or propionate. Five random faecal samples from each group were collected following observation of defaecation from 07:30h on Day-81. Samples were analysed by nLC-ESI-MS/MS (Orbitrap Elite) after in-gel sample preparation, trypsin digestion and tandem mass tag labelling. Data were assigned using Sequest HT engine to interrogate sequences in the Swissprot databases of bovine, barley and bacteria in Proteome Discoverer and for the latter focused on known ruminal and faecal microorganism orders (*Eubacteriales*, *Bacteroidales*, *Spirochaetales* and *Verrucomicrobiales*). Protein differences between groups were calculated based on pairwise ratio, the hypothesis test was background-based t-test set up in the PD software. The criteria of differential abundance of proteins were log2(fold change) > 1 or < -1 and $p < 0.05$.

Results In total, 142 bovine proteins, 14 barley proteins and 1,025 bacterial proteins, a large proportion of which were genus *Clostridium*, were identified in the faeces. No significant effects of the treatment of barley on the host or barley proteins were found, however 27 microbial proteins were found to be differentially abundant between diets. Among

the latter were proteins related to gene expression and regulation such as RNA polymerase and metabolic enzymes such as enolase. In the bovine proteins, mucosal pentraxin, which is predominantly expressed in the colon, was found in high abundance. Enzymes such as carboxypeptidase A1 and aminopeptidase, proteins that inhibit protease activities including, alpha-2-macroglobulin and serpin A3-1, and proteins that are involved in inflammation such as complement C3 and polymeric immunoglobulin receptor were also identified among the top 20 highly abundant bovine proteins. Plant-derived protease inhibitors serpin-Z4 and serpin-Z7 were found in the faeces.

Conclusions There were no significant effects of ammonia or propionate treatment of barley on faecal bovine or barley protein but bacterial protein abundances were affected by the diet. The present study provided baseline information about the abundance of host, dietary and microbial proteins and will inform the development of our future studies to optimize diets for cattle.

P131

NanoLC-MALDI-TOF/TOF MS approach for the proteomic analysis of honeybee drone larvae

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Honeybees (*Apis mellifera*) are the world"s most important pollinators, but they are involved not only in food production. Bees may also be the direct food for humans and animals. It has been shown that they are an excellent source of proteins and other nutrients. Although common in some parts of the world, such as Asia, Australia and Central America, the consumption of insects is still gaining popularity in Europe. However, to ensure the safety of consumers, the composition of bee larvae must be thoroughly investigated. Therefore, in this research project, we analyzed the proteomic content in honeybee larvae samples collected in Poland.

The qualitative analyses of bee drone (a male bee) larvae were performed using the nanoLC-MALDI-TOF/TOF MS (nano-liquid chromatography - matrix-assisted laser desorption/ionization - time of flight/time of flight mass spectrometry) platform (Bruker Daltonics). Before MS analyses, samples were extracted with water and pretreated with *ProteoMiner* (Bio-Rad) enrichment kit, which is based on a combinatorial hexapeptide ligand library. Then, the obtained extracts were subjected to tryptic digestion and, subsequently, desalted, purified and concentrated with *ZipTip* (Millipore) solid-phase extraction micropipette tips.

The proposed methodology resulted in the identification of proteins and peptides contained in bee drone larvae. Some of them have never been detected in this complex product before. The obtained results indicate that the majority of proteins included in the larvae body are enzymes. The other large group involve proteins participating in cellular respiration. They are antioxidant proteins, mitochondrial proteins, and proteins involved in oxidative phosphorylation, glycolysis, and citric acid cycle. The presence of enzymes, respiratory and ribosomal proteins indicates intensive larval development when many new biomolecules are synthesized. This stage requires vast amounts of energy, so it is not surprising that proteins involved in cellular respiration are present in the larvae

Based on the abovementioned results, it can be concluded that honeybee larvae are a rich source of proteins. Proteomic analyses can provide valuable information on the type and functions of proteins present in this complex natural product. To broaden the knowledge of the drone larvae composition, in the next step, we aim to identify the proteomic features of bioactive and nutritional properties, as well as those of toxic, allergenic, and harmful activities. In addition to increasing the safety of eating insects, this research may be the first step towards an improved understanding of the physiological processes occurring in the bee larvae body.

Acknowledgements: This project received financial support from the Polish National Science Centre (grant number 2016/23/D/NZ7/03949).

P132

Analysis of intact proteins with capillary zone electrophoresis coupled to mass spectrometry using uncoated and coated capillaries

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Objective: Proteins are the actuators of many vital biological processes. Understanding the structural

characterization, identification of macromolecules in their intact states therefore entails the studies of cell biology, disease prevention and treatment [1]. Top-down mass spectrometric (MS) technique is sensitive enough to enable the studies for structural and dynamical identification of intact proteins when coupled with capillary zone electrophoresis (CZE) [2]. However, a serious concern is the analyte adsorption on the bare fused silica capillary surface, which necessitates the application of extreme pH or the use of coatings to minimize the analyte-wall interactions [3,4]. Our study therefore involves the use of fused silica capillaries employing the background electrolytes with very low pH and compares the analytical performance with those which were coated with polybrene as a dynamic and linear polyacrylamide (LPA) as a static coating material.

Methods: The novelty of the recent work is to demonstrate the differences in the ideal operating conditions (optimal pH, proper capillary conditioning etc.) of each capillary by using CZE-MS.

Results: The results suggested that the analysis in bare silica capillaries resulted in good precision (0.56-0.78 RSD% and 1.7-6.5 RSD% for migration times and peak areas respectively) and efficiency values with minimum adsorption into the capillary surface. Coated capillaries showed higher resolving power for the separation of different forms (subunits of hemoglobin) of the protein. However, the separation performance in LPA coated capillary distinguished from other capillaries based on their stability, reproducibility over 25 runs and shorter analysis time in less than 10 min. The applicability of the proposed methods was also supported by the analysis of protein rich samples (e.g., snake venom).

Conclusion: Hereby, it could be concluded that the application of bare silica capillaries for the analysis of intact protein mixtures would be also an efficient choice compared to coated capillaries when ideal conditions are applied.

Disclosure of conflict of interest:

No potential conflict of interest was reported by the authors.

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P133

Combined top-down and bottom-up proteomics for the identification of small open reading frame encoded proteins

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Aim: Short open reading frame-encoded proteins (SEPs) represent a largely unexplored region of the proteome. The identification of SEPs has primarily been pursued via bottom-up proteomic analyses, however, certain characteristics of SEPs (e.g. low number of proteotypic peptides, novel N- and C-termini) make such identifications challenging. Top-down proteomic workflows allow direct identification of intact proteoforms, which enables the characterization of both post-translational modifications, and potentially novel N- and C- termini. Despite this, the efficient separation of analytes prior to top-down proteomic workflows remains a challenging limitation.

Method: SEPs and proteins below ca. 20 kDa (i.e. the low molecular mass proteome), are selectively isolated, firstly via a partial depletion/precipitation of

higher molecular mass proteins in 5% formic acid, and secondly, by retention of the SEPs and low molecular mass proteins via solid phase extraction. Subsequently, depletion of singly charged analytes is facilitated through strong cation exchange (SCX) chromatography. The resultant low molecular mass proteome is analysed with top-down LC-MS on an Orbitrap Fusion Lumos, utilising both HCD and EThcD ion activations, to further aid in proteoform characterisation. Bottom-up analysis of the samples was performed in concert to support top-down data interpretation.

Result and Conclusion: The use of both multiple enrichment strategies and multiple ion activation types, in combination with both top-down and bottom-up proteomic workflows, allows for a higher degree of confidence in the validity of SEPs that are identified. Employing this strategy allowed for the top-down characterization of 36 proteoforms mapping to 12 SEPs from the archaeon *Methanosarcina mazei* strain Gö1, with the concurrent detection and identification of several post-translational modifications.

P134

FAIMS with internal CV stepping for improved identification of proteoforms in top-down proteomics

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Objective

For the high confidence identification of proteoforms and an increased proteome coverage, fractionation prior to mass spectrometric (MS) analysis is a crucial step in top-down proteomics. Gas-phase fractionation strategies such as field asymmetric ion mobility spectrometry (FAIMS) have been shown to be highly beneficial for that purpose. However, the need for multiple injections using different compensation voltages (CVs) leads to a huge increase in measurement time and the amount of sample required. Therefore, we here investigated the use of internal CV stepping for single shot TD analysis, i.e., the application of multiple CVs per acquisition [1]. In addition, MS parameters were optimized for the individual CVs since different CVs target certain mass ranges.

Methods

Lysates of Caco-2 cells were analyzed after SPE based enrichment [2], MWCO treatment or GELFrEE fractionation of the low-molecular fraction (i.e. below 30 kDa) of the proteome using LC-MS (Fusion Lumos) either with or without FAIMS. Single CV-FAIMS data were compared with different combinations of multiple CVs.

Results

In accordance with earlier results [3] we found that lower (more negative) CVs favored the identification of lower mass proteoforms, while more positive CVs showed a bias toward higher molecular weight proteoforms.

To obtain both a uniform mass distribution and a high number of proteoforms (~1,700), the combination of four CVs (60, 50, 40, 20 V or 60, 50, 40, 0 V) was found the most appropriate, as significantly more proteoforms were identified in all mass ranges compared to w/o FAIMS.

Due to the correlation between CV values and mass range, we were able to adjust the measurement conditions, using optimized resolution and number of microscans depending on the particular CVs. We investigated the optimal combination and number of CVs for different gradient lengths and, validated the optimized settings with the low-molecular weight proteome of Caco-2 cells obtained using a range of different sample preparation techniques.

Compared to measurements without FAIMS both the number of identified protein groups (+60-94%) and proteoforms (+46-127%), and their confidence were significantly increased, while the measurement time remained identical. In total, we identified 684 protein groups and 2,675 proteoforms from CaCo-2 cells in less than 24 hours using the optimized multi-CV method.

Conclusion

Our data show that measurement with internal CV stepping leads to a significantly higher number of identified proteoforms compared to measurements w/o FAIMS.

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P136

Automated sample preparation of patient plasma samples via filter-added sample preparation (FASP) using the robotic platform Biomek i7

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Objective: As part of the DIASyM research core, located at the University Medical Center (UMM) in Mainz, our group focuses on the data-independent mass-spectrometry based proteome analysis of heart failure (HF) patient plasma samples compared to healthy controls. HF is the leading cause for hospitalization of individuals over 65 years of age. But until today, the pathophysiological mechanisms leading to HF are not completely understood. Our aim is to identify typical biomarkers for HF or those that are predicting a worsening of HF, as well as to better characterize the different subtypes of HF via deep phenotyping. Therefore, samples need to be prepared in a reliable, standardized, high-throughput manner using a robotic platform (Biomek i7, Beckman Coulter©). This enables sample preparation of up to 90 samples per day/360 samples per week. To establish and improve the automated sample preparation with Biomek i7, plasma samples of a small study cohort (EmDIA) were used.

Methods: A Biomek i7 (Beckman Coulter©) with an attached positive pressure ALP (Amplius) was used for sample preparation. Samples were digested using the filter-added sample preparation (FASP) protocol. The manual FASP-protocol was automated and improved for plasma digestion using Biomek i7 with samples derived from the EmDIA study (patient cohort with approximately 420 samples) and a plasma pool as control. Conditions of the FASP protocol were adjusted and experiments comparing the manual and automated method were performed.

Results: Washing-/incubation-conditions, pressure settings and liquid handling steps were optimized for the automated sample preparation, to reach the same or higher levels of identified peptides/proteins as the manual digestion protocol. Afterwards, the EmDIA samples were all prepared using the optimized automated sample preparation method. Reproducibility within one 96 well plate and within the whole study cohort was tested.

Conclusion: Automated sample preparation is essential to speed up and standardize high-throughput analyses. After in depth optimization, including pressure settings, buffer conditions and input amounts, we have successfully established a 96-well FASP-protocol for efficient processing of patient plasma samples. In contrast to manual processing, the automated protocol on a Biomek i7 minimizes hands-on time and enables routine high-throughput sample preparation. In combination with data acquisition on a timsTOF Pro 2 platform using diaPASEF with DIA-NN data processing, the workflow enables the quantification of typically around 350 proteins in 15 min instrument time from human plasma samples.

DIASyM (Data-Independent Acquisition-based Systems Medicine); FASP (filter-added sample preparation); MS (mass spectrometry); HF (heart failure), ALP (Automated Labware Positioner)

P137

Autoantigenomics in Neurology: Systemic 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" as a subset of the proteome) 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 non-responders. 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.

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Experiments with a deep learning transformer architecture for retention time prediction in mass spectrometry-based proteomics

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Introduction

Machine learning is playing an increasingly important role in analysis of mass spectrometry (MS)-based proteomics data. It has been shown that deep learning approaches are able to model variable-length peptide sequences similar to methods employed in natural language processing (Gessulat 2019; Yang 2020; Wen 2020). We aim to predict retention time through liquid chromatography (LC) of peptides in a typical LC-MS/MS experiment. A recent breakthrough in natural language processing is the transformer architecture that enables parallel processing with high accuracy (Vaswani 2017; Devlin 2018). The architecture also demonstrates state-of-the-art performance in other fields such as computer vision (Carion 2020) and biology (Rives 2021). Hence, it is highly desirable to experiment with the transformer architecture for our task.

Methods

We followed the original transformer architecture so that an efficient implementation can be readily available. Specifically, we implemented a deep learning model based on the BERT transformer architecture (Devlin 2018). We compared the result to state-of-the-art methods for retention time prediction including Prosit (Gessulat 2019), DeepDIA (Yang 2020) and AutoRT (Wen 2020). The three methods employ various alternative deep network architectures. The datasets in the three methods are used for training and validation of the transformer model.

Results

The performance of the transformer architecture is competitive with Prosit, DeepDia and AutoRT on datasets used in three respective methods.

Conclusions

The transformer architecture is suitable for modeling of peptide sequences for retention time prediction. In addition, we provide an open-source framework for evaluation of deep learning models for the research community.

P139

Proteome analysis of precursor lesions from pancreatobiliary cancer to improve early cancer diagnostic

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Objective:

Cell-subpopulation analysis becomes more important due to the big variants of different cell types in distinct tissue samples. Proteomic analysis of different tissue samples is important for the detection of proteins that are involved in tumor progression, with the potential to find new disease-specific biomarker to improve diagnostics. For pancreatobiliary (PB) cancer a specific biomarker for diagnostics is not yet known, except the tumor markers CA 19-9 and CEA, which are used for follow-up purposes but cannot be used for early diagnosis of PB cancer due to their elevation also in benign conditions, such as pancreatitis. The early stage in tumor progression is associated with a transition of normal cells into so-called precursor lesions, which seem to develop along similar pathways and show numerous morphological and molecular similarities. The group of Prof. Esposito at the Institute of Pathology developed methods for morphomolecular characterization of PB precursors as well as molecular subtyping of different precursor stages applied on formalin-fixed, paraffin-embedded (FFPE) tissues. For the characterization of PB cancer, several proteome studies have been attempted to detect proteins involved in progression. However, they provided only limited insight into the tumorigenic mechanism of PB cancer, because the results were biased by signals from different cell types such as acinar, endocrine, stromal and inflammatory cells. Here, we will optimize and apply sensitive methods combining microdissection and quantitative proteomics to get a deeper insight in the development of PB cancer.

Methods:

For label-free analysis of pancreatic laser microdissected FFPE tissue, a modified tissue lysis protocol with alternating freeze-thaw cycles followed by boiling and sonication steps are used to disrupt cells, reverse the formalin fixation and to extract proteins. For protein purification and processing for mass spectrometric analysis an optimized protocol for a single-pot solid-phase-enhanced sample preparation (SP3) method was applied to the FFPE lysates. Preventing possible protein loss, due to protein adherence on tube walls, the SP3 protocol has been reduced to a smaller scale by using PCR tubes with a compatible magnetic rack (caproMagTM) for magnetic bead handling.

Results:

Using these optimized methods, currently we are able to analyze 2.5 mm^2 FFPE pancreatic tissues (approx. 8,600 cells) and to identify around 2,300 proteins in high reproducibility. In 0.5 mm^2 FFPE pancreatic tissues (approx. 1,700 cells) it was possible to identify ~900 proteins covering still four orders of magnitude in intensity. To increase reproducibility and proteome coverage we aiming for TMT labelling considering a carrier proteome.

Conclusion:

Optimization in sample isolation and preparation of FFPE tissues can provide deeper insights on the proteome to identify possibly selective biomarkers for early diagnosis of PB cancer.

P140

A Fully Automated High-Throughput, Deep-Scale Quantitative Plasma Proteomics Workflow Enables Quantitively Profile More Than 1000 Proteins Per Sample

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Introduction:

LCMS based plasma proteomics is advancing our understanding of human molecular pathophysiology and empowering the discovery of therapeutic targets

and biomarkers. However, managing throughput, proteome depth and reliability altogether represents a major gap to fully enable meaningful large cohort proteomics studies. Those limitations include 1) variability from manual sample preparation, 2) low throughput using nano-flow HPLC, and 3) the caveats of managing quantitative accuracy, precision and dynamic range in the data to avoid compromising proteome depth.

Methods:

Here, we standardized a plasma profiling workflow solution using the following building blocks: 1) A liquid handler for automated sample preparation; 2) A next generation LC that enables higher robustness and peak capacity; 3) The unified integration with High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS™ Pro Interface) coupled to a Thermo Scientific™ Orbitrap Exploris™ mass spectrometer. 4) Furthermore, we fully benefited from a new search engine, CHIMERYS identification node, which uses deep neural networks to maximize the retrieval of protein identifications yielding significant improvement compared to conventional search engines.

Results:

To investigate the reproducibility and quantitative performance, plasma samples were processed by the entire workflow in triplicate. The correlations of the protein intensity between any of the two replicates are >0.990, demonstrating the excellent quantification precision of the workflow. We also tested how our optimized method performs as compared with standard DDA, the same plasma samples were analyzed by the standard DDA method in three technical replicates. We observed more than 100% increase in protein and peptide IDs, yielding over 1000 proteins from 1 µL plasma.

Conclusions:

In this work, we leverage automated sample preparation, a next generation low-flow UHPLC, FAIMS, advanced MS data acquisitions, and a novel search engine using deep learning, to maximize throughput and quantitative performance, allowing the quantitation of more than 1000 proteins per sample.

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Enhancements of MaCPepDB

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Objective

Often challanged with small amounts of samples, researchers who wants to run targeted protemic experiments like single-, multiple- and parallel reaction monitoring (SRM, MRM and PRM), need to now their targets before even measuring their samples once. Furthermore, it is important to know, whether selected peptides are unique, at least for the species of interested.

To these lengths we developed MaCPepDB (Mass Centric Peptide Database). MaCPepDB contains the tryptic in silico digest of all known proteins in UniProt, stored in an efficient manner to be quickly searched.

Methods

The focus of each search in MaCPepDB is based on the theoretical mass of peptides of interest and can be specified by additional parameters, like the mass tolerance, potential post translational modifications, taxonomical species or wether the peptide is contained in the reviewed SwissProt or computational annotated TrEMBL part of UniProt.

While the search for peptides by theoretical mass is the main purpose of MaCPepDB, MaCPepDB contains also links between peptides and the respective proteins, which makes it easy to look up the proteins from which a peptide originates and vise versa.

Through the provided searches and information, researchers are able to design SRM-, MRM- and PRM-assays for their experiments without spending sample amounts for exploratory measurements.

With laboratory researchers in mind, MaCPepDB provides a simple way to access the database via a web interface, which also provide several exports so the data can be imported in other software or archived for publications or reanalyses.

Each function of the web interface is also available via a web API, which makes it easy to include MaCPepDB in existing analyses and workflows.

Results

In the latest release of MaCPepDB we were able to increase the performance and stabilize the response times with the help of modern distributed database technologies, as a result the number of simultaneous users are improved.

This performance gain allowed us to provide additional data for each peptide. One of these is a list of taxonomies for each peptide, which highlights whether the peptide is unique or shared in each respective species.

Conclusion

MaCPepDB provides two easy and fast ways to search tryptic peptides in the complete UniProt KB. While the web API is useful for programatically access in workflows, the web interface can be used without any computational knowledge, making access easy for everyone without programming skills.

As MaCPepDB is entirely open source, it is also simple to setup a more specialized version of MaCPepDB for specific applications.

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Proteome profiling of non-muscle invasive and muscle invasive bladder cancer highlights distinct subgroups with unique proteome biology

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Urothelial bladder cancer ranks among the top ten malignancies worldwide with approximately 20% of cases presenting as a muscle-invasive entity with worse prognosis. We investigated the proteome biology of non-muscle invasive and muscle invasive bladder cancer (NMIBC / MIBC) with a cohort comprising 17 NMIBC and 51 MIBC cases. Protein was extracted from formalin-fixed, paraffin embedded (FFPE) samples after macrodissection and analyzed by data-independent acquisition (DIA), yielding an average proteome coverage of > 6000 proteins per sample. Comparison of MIBC vs. NMIBC highlighted an enriched proteome signature of extracellular matrix (ECM) and immune response components in MIBC together with depletion of lipid metabolism components. Moreover, semi-tryptic data analysis suggests elevated levels of proteolytically truncated proteins in MIBC, indicative of increased endogenous proteolytic processing. Unsupervised clustering of the MIBC proteomes produced three distinct clusters with signatures of metabolism, immune-functionality, and ECM. In correlation with histopathologically determined levels of immune cell infiltration (ICI), the metabolic cluster presents as rather immune desert. Cox regression

analysis suggests a tendency for prolonged progression free survival (PFS) for the metabolic cluster and shortened PFS for the immune cluster. In line with this observation, the metabolic subgroup clusters close to NMIBC in principal component analysis, indicative of proteomic similarities. Further, we are aiming to uncover non-annotated protein sequences and variants by proteogenomic approaches. In summary, our study provides a deep insight into the proteome biology of urothelial bladder cancer and its clinical relevance.

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Automated DIA data processing using DIA-NN within the PaSER environment

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Data independent acquisition strategies is now routinely employed for sensitive high-throughput quantitative proteomics. Powerful dia-PASEF strategy in particular allows for deep proteomes in short gradient times (<20 min) making use of ion mobility separation and thus making large cohort studies feasible on wider projects. DIA-NN is a novel software package that uses neural networks providing fast and sensitive quantitative information from the data. Here we integrate DIA-NN within the PaSER environment for a streamlined workflow for the analysis of many samples in a short analysis time with no file transfer or data migration.

Data were acquired on a timsTOF pro instruments with gradients ranging from 5 to 90 minutes. DIA-NN was modified to become CCS-enabled and process data in the most expedient fashion. The PaSER GUI was designed such that first-pass analysis is predefined automatically triggering quantitative analysis. Retrospectively, match-between-runs (MBR) analysis can be triggered on the whole project or subset of user defined experiments.

Human, Yeast and E. coli (HYE) digested mixtures at different but known ratios with injection loads from 50ng to 600ng were run at different gradient lengths in replicate resulting in >2500 proteins at short gradients to >9000 proteins identified and quantified at longer gradients. Quantitative accuracy was shown to be <20% CV. Using the DIA-NN as integrated into PaSER creates a seamless approach to dia-PASEF analysis.

Automated workflows for dia-PASEF using DIA-NN on PaSER streamlines experiments to results

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In-depth screening of plasma proteins from dried blood spots with proximity extension assay

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Dried blood spot (DBS) samples represent a unique individual sample with major advantages including the non-invasive capillary collection method and the long-term storage, also at room temperature. Originally designed for newborn screening of metabolism and congenital disorders, DBSs are successfully applied in clinical research for immunological studies. Indeed, serum antibodies and inflammatory markers are detected in DBSs with a comparable efficiency than in venous samples. The limited sample volume retrieved from the filter paper represents a limitation for many analytical assays. Olink®Explore is the newest high-throughput protein biomarker platform that uses Proximity Extension Assay (PEA) technology coupled to a readout methodology based on Next Generation Sequencing (NGS). The combination of four Explore panels allows detection of nearly 1500 proteins in plasma by using only 10 µl of specimen.

With the future perspective to screen a large number of samples from the GPPAD (Global Platform of

Autoimmune Diabetes) bioresource by using the Olink®Explore technology, we have conducted a pilot experiment on 24 DBS samples from the cohorts. Upon extraction of DBS samples, we ran three different sample dilutions on four Olink®Explore panels (Cardiometabolic, Inflammation, Oncology, and Neurology).

The aim of the study was to evaluate in the first place the detectability of the 1472 assays in DBSs compared to plasma samples. Hook- and matrix-interference effects were analyzed, as well as the technical reproducibility. We also investigated the influence of pre-analytical variations, such as storage temperature and time, as well as the influence of different sampling sites.

We concluded that DBS optimally performed in the Olink®Explore platform, with an assay detectability that nearly matches pure plasma and excellent reproducibility. Currently, we also explore the detectability of the extended portfolio of plasma proteins covered in the recently released Olink®Explore 3072 panels in DBS samples. Our positive results lay a solid foundation for applying DBSs in screening plasma proteins in large cohorts using the innovative Olink®Explore technology.

P145

Forensic proteomics: application in the present and potentials for the future

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Objective: Mass spectrometry (MS)-based analysis is a promising alternative for the identification and characterization of biological traces in forensic investigations. In contrast to established forensic tests, MS-based analyses exhibit high sensitivity as well as specificity and are outstanding due to their multiplex capability, which enables a combination of several test methods. Aim of our study was to establish an MS-based workflow for the identification of forensic relevant body fluids like blood, saliva, sperm and vaginal fluid in order to overcome limitations of the current forensic tests. In the case of mixed traces from more than one person, it is currently not possible to determine the origin/person of the respective body fluid (not even by DNA analysis). Therefore, it was furthermore our goal to develop an approach enabling body fluids from mixed traces to be assigned to specific individuals on the basis of individual information, such as gender or blood groups.

Methods: For MS-based analysis of forensic samples, various sample preparation strategies were investigated, including in-gel, in-solution and filter aided sample preparation-assisted digestion. For the identification of body fluids specific marker proteins were detected by a combined analysis using data-dependent acquisition (DDA) and data-independent acquisition (DIA) using the nanoHPLC system Ultimate 3000 coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific). Suitable body fluid specific peptides were selected and used to set up a targeted MS method with parallel reaction monitoring (PRM) for multiplex analysis of blood, saliva, semen and vaginal fluid.

Results: A pipeline for LC-MS-based identification of body fluids was developed for blood, saliva, semen and vaginal fluid. In order to develop a fast, robust and sensitive method specific peptides for each body fluid were selected and used to set up a targeted LC-MS method using PRM. In addition, defined classification criteria were specified for the assignment of the body fluid. The applicability of the PRM method and the classification criteria was examined in a blind study and showed an identification rate of 100%. Currently, the PRM approach is in a trail phase, in which forensic traces are analyzed. Strategies for individualized body fluid assignment are in progress and will be presented.

Conclusion: Our method enables unambiguous identification of body fluids from forensic traces showing the high potential of LC-MS for present and future forensic purposes

P147

Method for parallel high-throughput proteomics and transcriptomics of breast cancer samples and its potential impact in biomarker discovery

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OBJECTIVE

In Breast Cancer (BC), personalised medicine has become increasingly important, with the goal to identify responders, reduce side effects, and improve efficacy [1]. Thus, there is a need for new improved biomarkers to tailor treatment to patients. Omics strategies have been applied in BC, mostly genomics and transcriptomics, describing the "intrinsic" subtypes. Proteomics has the advantage of more closely reflecting the phenotype, but most studies depend on peptide fractionation and Data Dependent Acquisition (DDA) mass spectrometry (MS) analysis, increasing instrument time and giving lower quantitative accuracy [2]. Here, we present a high-throughput proteomic method that can be used in parallel with RNA-sequencing for precision medicine.

METHODS

To enable RNA and protein measurements, we developed a semi-automated sample preparation workflow based on flowthroughs from RNA extraction. We applied a Data Independent Acquisition (DIA) MS approach on 67 samples from the Sweden Cancerome Analysis Network – Breast initiative [1] and processed the data using EncyclopeDIA [3] DIA-NN [4]. To assess the analytical quality of the methodology, we acquired the data using DDA and compared them to next generation RNA-sequencing data from the same samples [5]. We then calculated correlations between the different datasets and compared differentially abundant proteins between subgroups based on clinical parameters.

RESULTS

When correlating DIA to RNA-seq data, our results show a median Spearman correlation of approximately 0.5. After removing RNA/protein pairs with low variance across samples, the median correlation was 0.62. In comparison, correlating RNA-seq data to DDA yielded a median value of 0.44. Overall, these correlations are higher than previously reported [2, 6], demonstrating the positive impact of the herein proposed acquisition method.

The higher correlation showcases the relevance of transcriptomics. Nonetheless, it is important to mention that proteomics methods have the possibility of capturing proteoform information, e.g., post-translational modifications, adding a unique layer of information. Besides, certain functional protein groups associated with, e.g., inflammation, angiogenesis, and oestrogen response were reported to correlate less well with their RNA counterparts [6].

CONCLUSION

The results substantiate the use of DIA proteomic approaches, including processing via DIA-NN, to study BC. Analysis of larger cohorts could enable the further development of already existing subtyping, as well as provide useful insight into BC, potentially contributing to precision medicine.

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P148

Inclusive Quantification Assay of Wider Des-γ-Carboxyprothrombin Proteoforms using Targeted Mass Spectrometry for Surveillance of Hepatocellular Carcinoma

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Objective:

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. Des-gamma-carboxyprothrombin (DCP or PIVKA-II) is a serological surveillance marker for HCC, resulted from an impaired carboxylation process at glutamic acid residues within the N-terminal domain. DCP exists in the blood as a

mixture of proteoforms with different degree of carboxylation states at N-terminal domain (Gla domain). The heterogeneity of DCP may affect the accuracy of measurements using an immunoassay that relies on antibody reactivity to an epitope. The object of the present study is to improve the DCP measurement assay by applying a mass-spectrometry (MS)-based approach for more comprehensive quantification of DCP proteoforms.

Method:

We examined potential non-carboxylated peptides (which we will refer to as "Glu-peptides") within the various des-carboxylation states of DCP Gla domain. The multiple reaction monitoring-MS (MRM-MS) assay was developed to quantify multiple Glu-peptides for the inclusive quantification of DCP proteoforms. We performed the MRM-MS assay in 300 patients consisted of 100 HCC patients and 200 at-risk patients. The diagnostic panel was constructed by stepwise backward logistic regression with 10-fold cross-validation (100 times repeated). The diagnostic performance of the panel was validated at an external cohort consisted of 184 HCC patients and 134 at-risk patients.

Results:

The area under the receiver operating characteristic curve (AUROC) values for the quantitative MRM-MS assay were 0.743 and 0.742 in the training and test sets, respectively, whereas those for the immunoassay were 0.743 and 0.704. The DCP 3-Glu-peptide panel had statistically equivalent AUROCs to those of the immunoassay, based on DeLong`s test ($P > 0.05$). We combined the DCP panel with serum AFP levels to enhance the predictive power for HCC detection by further logistic regression analyses. The combined model using both the 3-Glu-peptide panel and serum AFP levels increased the AUROC values to 0.903 (95% CI, 0.855–0.952) for the training set, which outperformed serum AFP levels [AUROC = 0.770 (95% CI, 0.698–0.842)], based on DeLong`s test ($P < 0.05$). In the test set, the AUROC value of the combined model significantly also increased, from 0.844 to 0.913 (95% CI, 0.851–0.974). Notably, the combined model had greater sensitivity in both the training and test sets (68.9% and 76.7%, respectively), whereas the serum AFP levels alone had low sensitivity (35.7% and 56.7%, respectively). The MS-based DCP assay was validated using an independent validation set. The AUROC values of the 3-Glu-peptide panel and the combined model with AFP levels were 0.793 (95% CI, 0.745–0.842) and 0.863 (95% CI, 0.822–0.903), respectively, for the independent validation set.

Conclusion

The MS-based quantitative DCP assay is superior to antibody-based quantification due to the cost-effectiveness and high reproducibility with equivalent performance.

P149

Proteomics characterization of interferon α subtypes for the better understanding and treatment of virus infections

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Objective

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 infections, such as HIV and COVID-19, induce different expression patterns of IFN-α subtypes, resulting in the expression of variable restriction factors, causing different immunological outcomes. However, the exact mechanisms underlying the anti-viral activities of individual IFNα subtypes are not completely understood. Therefore, the exact biological functions and differences within IFN-I downstream signalling induced by IFN-α subtypes needs to be studied. To investigate these variations, as well as the therapeutically relevant effects, it is important to accurately quantify the individual IFN-α subtypes. So far, the high sequence similarity hindered development of immunological assays and the quantification of the individual subtypes.

Poster presentation

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. Second, to understand the biological functions of the subtypes, the downstream signalling that are triggered by the individual IFN-α subtypes were investigated using phosphoproteomics. Therefore, peripheral blood mononuclear cells (PBMCs) were isolated and treated with two out of the 12 different IFN-α subtypes and changes in the phosphorylation were analysed.

Results

Calibration curves were generated for all peptides and the lower limits of quantification were determined in complex matrices. Using the PRM assay, we were able to quantify spiked-in recombinant protein concentrations of interferon alpha subtypes in THP-1 cell conditioned culture medium. Secondly, changes in the phoshpoproteome were detected between different IFN-α subtypes which might provide novel insights into the anti-viral activity of the subtype IFN-α2 and IFN-α14.

Conclusions

Taken together, we aim to determine the concentrations of IFN-α subtypes in a variety of sample types using PRM. We will study the different IFN expression patterns between experimental groups and define the relation between the subtypes and the antiviral outcome. At the same time, the assay presented here may have the potential to be used in clinical settings to measure the IFN-α profiles of individual patients. This will allow new insights into antiviral host reactions, disease progression and could then support the customized medication of patients after virus infection.

P150

Quantification of contactins in cerebrospinal fluid samples of dementia patients

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Objective

Contactins are six structurally related proteins (CNTN1-6) and play a critical role in brain function by modulating several neuronal activities. Differential expression of contactins has been observed in neurological disorders. Therefore, we aim to evaluate the power of contactins as biomarker candidates for distinguishing different types of dementia disorders, i.e., Alzheimer"s disease (AD), frontotemporal dementia (FTD), Lewy body dementias (LBD) and non-neurodegenerative controls. Due to their very low abundance in clinical samples, highly sensitive measurements are required but are not available to date. Thus, we want to establish a mass spectrometry-based targeted proteomics method for the determination of contactins.

Methods

We implemented targeted proteomic analysis to quantify contactins in cerebrospinal fluid (CSF) samples collected from 100 dementia patients and non-neurodegenerative controls. The biobank center of Ulm University Hospital provided all patient samples. For digestion 200 µL CSF sample was used. The generated tryptic peptides were fractionated by centrifugation through strong cation exchange STAGE-Tips into 6 fractions. Each time almost 75% (20µL) of the fractionated sample was injected into a C18 PepMap100, 5 µm, 0.3 × 5.0 mm trap column using an Agilent 1260 HPLC system operating at a flow rate of 200 µL/min. An Eksigent MicroLC200 chromatographic system was used to separate peptides in an Eksigent HALO Fused-core C18, 2.7 µm, 0.5 × 100 mm analytical column. Thereby, within an LC-MS run time of 15.5 minutes, a very short gradient of 10 minutes and a flow rate of 15 µL/min were applied. Ionized peptides were analyzed on a QTRAP 6500 mass spectrometer. Skyline

software was used for data evaluation and calculation of the light/heavy ratio of analyzed peptides.

Results

A multiple reaction monitoring (MRM) method was developed for the quantification of contactins in CSF samples. Isotopic labeled standard peptides (QPrESTs and AQUA peptides) were used for method development, and the final method consists of 13 peptides derived from all six contactins (CNTN1-6). The preliminary results show a good sensitivity of the method within the pg/mL concentration range. The developed MRM method will be applied to a cohort of CSF samples from patients with AD, FTD, LBD and controls. The level of contactins will be assessed and compared between patient groups included in this study.

Conclusion

We developed an MRM method for the sensitive and accurate determination of contactins in CSF samples.

P151

Unbiased Antimicrobial Resistance Detection from Clinical Bacterial Isolates Using Proteomics

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Objective

Antimicrobial resistance (AMR) poses an increasing challenge for therapy and clinical management of bacterial infections. Currently, AMR detection relies on phenotypic assays, which are performed independently from species identification. Sequencing-based approaches are possible alternatives for AMR detection, although the analysis of proteins should be superior to gene or transcript sequencing for phenotype prediction as the actual resistance to antibiotics is almost exclusively mediated by proteins. In this proof-of-concept study, we present an unbiased proteomics workflow for detecting both bacterial species and AMR-related proteins in the absence of secondary antibiotic cultivation within

Results

Our tailor-made data analysis concept for bacterial proteomics [1] is based on a newly developed software (rawDIAtect) for prediction and reporting of AMR from peptide identifications. It was validated using a cohort of 7 bacterial species and 11 AMR determinants represented by 13 protein isoforms, which resulted in a sensitivity of 98% and a specificity of 100%.

Conclusion

Our workflow is based on rapid and universal sample preparation of bacterial isolates using SPEED [2] and enables species identification and detection of AMR determinants in an unbiased fashion from data-independent acquisition (DIA) MS data with high sensitivity and specificity. MS1 peptide features were used for bacterial species identification [3]. This information is then used to predict sample specific peptide libraries in silico and therefore enable detection of AMR determinants. This strategy eliminates the need for searching large sequence databases, which is a major limitation for proteotyping bacteria. In the past few years, improvements of LCMS technologies towards rapid analysis of proteomes have been published. The presented workflow could be adopted to these technologies without any further adaptation, which would increase throughput for species identification and AMR detection dramatically. This could turn proteomics into a serious contender for genomics with regard to the prediction of bacterial phenotypes from molecular data.

1 Blumenscheit C., et al., Unbiased Antimicrobial Resistance Detection from Clinical Bacterial Isolates Using Proteomics. Anal Chem,2021.**93**(44): p.14599-14608 https://pubs.acs.org/doi/10.1021/acs.analchem.1c00594

2 Doellinger J., et al., Sample Preparation by Easy Extraction and Digestion (SPEED) A Universal Rapid and Detergent-free Protocol for Proteomics Based on Acid Extraction. Molecular & Cellular Proteomics,2020.**19**(1): p.209--222

3 Lasch P., et al., Identification of Microorganisms by Liquid Chromatography-Mass Spectrometry (LC-MS(1)) and in Silico Peptide Mass Libraries .Mol Cell Proteomics,2020.**19**(12): p.2125-2139

Fig1: Table of content

Fig2: Overview of the rawDIAtect workflow and programs used

Poster presentation

Fig. 1

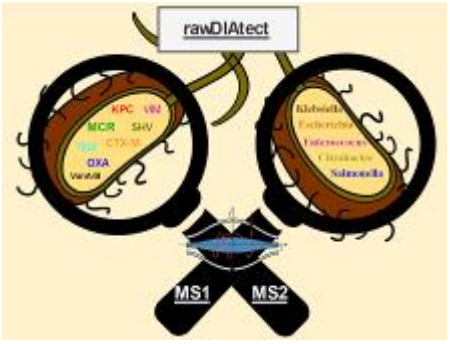
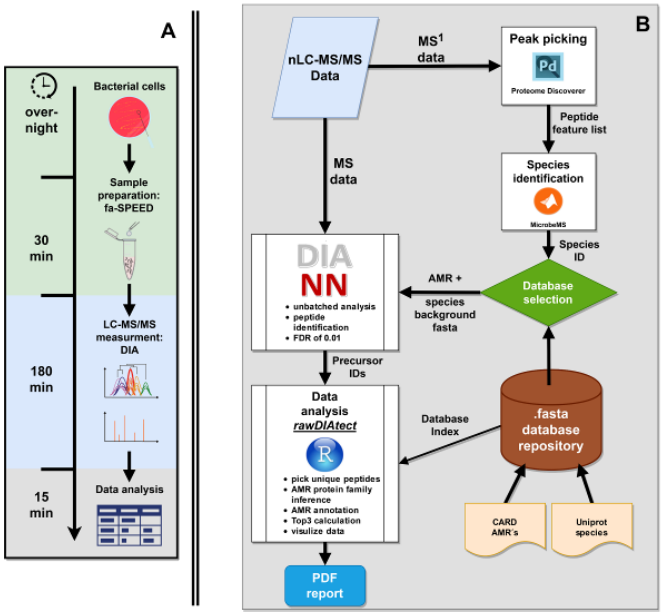


Fig. 2



P152

Examination of amino acid and biogenic amine content of serum and tear samples by UHPLC-MS

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Objective: Omics techniques are gaining prominence in several areas of scientific research, and metabolomics approaches are increasingly used, as the amount of different metabolites may be indicative for some pathological changes, such as obesity or diabetes. In this study, an ultra-performance liquid chromatography mass spectrometry method was developed to simultaneously determine 23 amino acids and 10 biogenic amines in 3 matrices (water, tear and serum).

Methods: Totally, 23 amino acids including His, Asn, Ser, Gln, Arg, Gly, Asp, Glu, Thr, Ala, Pro, Cys, Lys, Tyr, Met, Val, Ile, Leu, Phe, Trp, Tau, Cit and Orn; and 10 biogenic amines such as histamine, ethanolamine, methylamine, ethylamine, putrescine, serotonin, cadaverine, tyramine, tryptamine and phenethylamine were analyzed by an Acquity H-Class UPLC system (Waters) and a 5500 QTRAP (ABSciex) mass spectrometer. We have developed an 11 minutes chromatographic gradient including re-equilibration. The samples were derivatized with AccQ-Tag derivatization kit (Waters). The AccQ-Tag Ultra reagent (6-aminoquinolyl-N-hidroroxysuccinimidyl carbamate) converts both primary and secondary amino groups to stable derivatives.

Results: We determined the linear dynamic range, limit of detection, limit of quantification, intra- and interday accuracy, precision, freeze-thaw stability and matrix effects in the three applied matrices. We also compared the amino acid and biogenic amine content in tears and serum samples from healthy individuals.

Conclusion: In conclusion, we have developed a rapid, sensitive and accurate method for the simultaneous analysis of 33 biomolecules in complex biological samples.

Acknowledgement: The research was funded by NKFIH FK 134605 and GINOP-2.3.4-15-2016-00002.

P153

Consistent and sensitive absolute quantification of proteins in liquid biopsies by FastCAT workflow

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Objective

Absolute (molar) quantification of disease marker proteins in liquid and solid biopsies is a key method of clinical proteomics. Workflows based on concatenating multiple quantotypic (Q-)peptides in a metabolically labeled chimeric protein (CP) standard offer excellent multiplexing, but are cumbersome and have limited dynamic range since the proteolysis produces peptides in equimolar concentration. In this work, we developed rapid, sensitive and flexible FastCAT workflow that

allows parallel molar quantification of dozens of proteins with better that 100-fold dynamic range and CVs<15%.

Methods

Heavy labeled (13C6,15N4-arginine and 13C6-lysine) CP standards were expressed in E. coli. All CPs were composed of five reference (R-)peptides (for in-sample determination of CP amount based on spiked-in reference protein, BSA) as well as multiple Q-peptides per each quantified target protein. 20 µL of cerebrospinal fluid (CSF) samples from patients with multiple sclerosis were spiked with CPs and BSA, and in-solution digested with trypsin/Lys-C (enzyme-to-substrate ratio, 1:20). Protein digests were analyzed by nanoflow RP-LC-MS/MS with scheduled parallel reaction monitoring (PRM) method on a Q Exactive HF mass spectrometer. Data were processed and analyzed using Skyline.

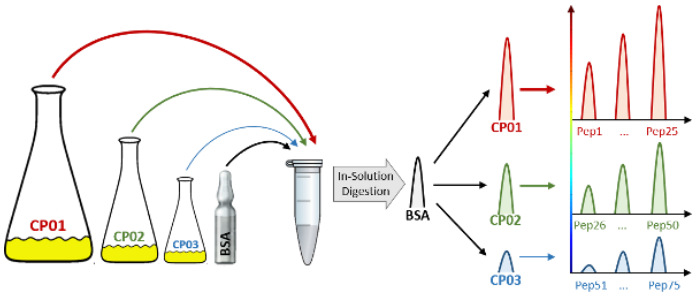
Results

Multiple, short (<50 kDa) and unpurified CP standards were used to quantify 10 neurological marker proteins in CSF samples in a single LC-MS/MS run. Controlled scrambling of R-peptides used for in-situ certification of the CPs amount enabled high degree of multiplexing. Using multiple CPs, whose amounts were adjusted to match the abundance of target proteins, increased the dynamic range (from 20 to 2000 ng/mL for chitinase-3-like protein 1 and apolipoprotein E, respectively). We achieved the precision of 5% (median CV). Proteins were independently quantified by 2 to 4 proteotypic peptides with concordance of 12% (median CV). Determined protein concentrations corroborated previous reports by SRM and PRM and were much more consistent than ELISA.

Conclusion

FastCAT workflow supports accurate, sensitive and consistent absolute quantification of proteins in liquid biopsies.

Fig. 1



P154

Automated, Rapid, Parallel Protein Extraction for Analysis of Low Input FFPE, Fresh Tissue and Cells Clinical Samples

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Question: Translational or clinical laboratories require automated, hands-off solutions for protein sample preparation to provide reproducibility, increased efficiency, higher quality results and faster turnaround time. This presentation features protocols using "Adaptive Focused Acoustics (AFA)" for single-pot, simultaneous multi sample processing from diverse inputs in a convenient 8-strip format. Solutions include non-toxic protein extraction from Formalin-Fixed Paraffin-Embedded (FFPE) tissue, fresh frozen tissue, and cells for mass spectrometry-based (MS) proteomics using short gradient runs.

Methods: Protocols are perfectly suited for batches of 40 samples per day and were adapted from previous published work on higher throughput approaches. Those methods work in small volumes (75ul or less): they generally use SDS based buffers and SP3 as the clean-up and digestion method. Other buffers/clean-ups can be used. For FFPE, paraffin is emulsified without toxic solvent, and removed during the SP3 washes, in a single pot process. 5 micrograms of peptides were run on a 5 min gradient using scanning Swath and for data analysis, DIA-NN was used with an in-house project specific spectral library.

Results: The employed protein extraction and analysis workflow displays highly consistent and reproducible results for the various sample inputs tested. Peptide and protein identifications are particularly good for short gradients and average compared to conventional 2h gradient analysis. The extraction process is fully compatible with a single pot approach.

Results were analysed at 1%FDR. For all sample types, variance distribution across protein groups is very conserved between replicates:

- For Human liver FFPE samples, around 15,000 peptides and 2,500 proteins groups were found, with a median CV of 13.7%, in consistency with previous studies. Pearson correlations across all proteome measurements were high with values above 0.96, showing high workflow reproducibility.
- For Fresh Rat liver, around 17,500 peptides and 2,250 proteins groups were identified. Reproducibility was high with median CV found at 15%. Like in the FFPE experiments, the consistency of identification between replicates was very good with all Pearson correlation values above 0.97.
- For cultured HeLa cells, around 30,000 peptides and 4,000 proteins groups were identified. Median CV was calculated at 13.9% with an overall Pearson correlation above 0.96, which was very satisfactory with regards to the gradient length, and compatible with other studies.

Conclusion:

We developed a robust preparation workflow enabling reproducible mid-sized studies in pre-clinical and clinical research. This fast, efficient, and consistent mid-throughput approach is ideal for sample series of 10 to 40 samples, including for

- Samples from the clinic, such as fresh frozen tissue material,
- Samples from pathology or biobanks such as PFA, FFPE or DBS samples,
- Targeted assays for marker protein identification.

P155

Serum proteome profiling of intrahepatic cholangiocarcinoma towards diagnostic biomarker identification

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Introduction

Intrahepatic cholangiocarcinoma (iCCA) is the second most common subtype of liver cancer, fourth leading cause of cancer related deaths worldwide, with incidence rates rising faster than for any other cancer. With a dismal 5-year survival rate of solely 17%, iCCA has a hopeless prognosis explained by its refractory nature and late diagnosis, hindered by its early phase asymptomatic makeup. The current diagnosis is the tumor biopsy (sensitivity 74.6%, 12.5% in hepatocellular carcinoma suspected patients). As such, there is an urgent need for the development of an accurate diagnostic tool, one encouraging option being a proteomic-based biomarker signature.

Objective

Thus, the aim of this study was to explore the serum proteome of iCCA patients in comparison to hepatocellular carcinoma (HCC), chronic hepatitis (CH), liver cirrhosis (LC) and healthy controls (HC) by employing mass spectrometry techniques.

Poster presentation

Methods

Blood samples were collected from 60 patients (15 iCCA, 15 HCC, 15 CH, and 15 LC) and 15 HC. After depletion of six highly abundant proteins, serum samples were subjected to label-free ultra-high-performance nano-LC coupled with ultra-high definition Q-TOF mass spectrometry (nano-LC-UDMSE) proteomics analysis. Raw data were acquired using MassLynx™ Software and data was processed by using ProgenesisQI for proteomics (Waters Corporation). Only proteins that could be identified in >75% of all samples from a group were considered for further analysis.

Results

Based on the MSE spectra and protein intensities of the individually analyzed samples, the serum proteome was characterized. Approximately 300 non-redundant proteins were identified in each sample with good coefficient of variation (CV<15%). Several proteins sets that could aid iCCA diagnosis, but also differentiation of iCCA from HCC, CH and LC were identified and comprise potential biomarker signatures. A set of proteins was selected for further validation using complementary methods, such as ELISA, in an independent cohort.

Conclusion

This is the first study to employ UDMSE detection towards serum proteome characterization of iCCA in comparison to HCC, CH, LC and HC. Unique biomarker signatures based on proteins sets were identified and could be further used towards iCCA diagnosis. Furthermore, a set of proteins was selected for further validation in an independent patient sample cohort. We consider iCCA clinical management directed towards a protein-based biomarker signature validation and clinical implementation a crucial opportunity.

Acknowledgements

Romanian National Ministry of Research, Innovation and Digitalization: Postdoctoral Research Project PN-III-P1-1.1-PD-2019-0852/PD113 awarded to Maria Ilieș.

P156

Quantification of C-terminal alpha-1-antitrypsin peptides by LC-MS/MS: A novel tool for characterizing systemic inflammation

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C-terminal peptides (CAAPs) of the highly abundant serine protease alpha-1-antitrypsin (A1AT) have been identified at various lengths in several human materials and have been proposed to serve as putative biomarkers for a variety of diseases. Those CAAPs are enzymatically formed and these enzymatic activities are often associated with excessive immune responses (*e.g.* sepsis, allergies). However, most of those CAAPs have been either detected using *in vitro* incubation experiments or in human materials which are not easily accessible. To gain a comprehensive understanding about the occurrence and function of CAAPs in health and disease, a LC-MS/MS method for the simultaneous detection of nine CAAPs was developed and validated for human plasma (EDTA and lithium-heparin) and serum. Using this newly developed screening method, we were able to detect and quantify five CAAPs in healthy individuals thereby providing an initial proof for those five peptides present in human blood. Concentrations of four CAAPs in a clinical test cohort of patients suffering from sepsis were significantly higher compared to healthy controls. These results reveal that in addition to C42 other fragments of A1AT seem to play a crucial role during systemic infections. The proposed workflow is simple, rapid and robust; thus this method could be used as diagnostic tool in routine clinical chemistry as well as for research applications for elucidating the diagnostic potential of CAAPs in numerous diseases.

Poster presentation

P157

Extracellular Matrix Enrichment In Lesions from Patient Material After Total Hip Arthroplasty

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Question: Osteolysis is a pathological process in which the release of microscopic plastic debris from a prosthetic joint initiate an innate immune response. Currently the only treatment is revision surgery. Previously, a tissue-specific biological efficacy for Denosumab on osteoclast number in the treatment of osteolysis could be demonstrated. The aim of this study was to conduct a comprehensive proteomic analysis of osteolysis tissue, comparing Denosumab treatment to placebo patient material. We evaluated a sequential sample preparation protocol with a special focus on enriching the extra cellular matrix (ECM) as source of changes introduced by the treatment of osteolysis.

METHODS: Tissue was collected from each study participant as they underwent revision surgery at week 8 post intervention. Protein was isolated using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen). Protein pellets were prepared for mass spectrometry in two steps. First, protein solubilized in RIPA buffer was digested using a filter-aided sample preparation protocol. The second approach was focused on unlocking proteins not yet solubilized as well as on enriching ECM in the samples. To this end, the remaining pellet was cooked in guanidinium chloride and precipitated, followed by in-solution digestion. Peptides were analyzed using quantitative LC-MSMS shotgun proteomic on a QExactive HF-X coupled online to a Dionex RSLC (Thermo Fisher). Data from different sample preparations were analyzed separately as well combined as fractions using Proteome Discoverer Software (Version 2.4, Thermo Fischer). Ingenuity Pathway Analysis (IPA, Qiagen) was used to identify pathway enrichment.

RESULTS: Over 3500 proteins were identified and quantified in the combined fractions, mainly originating from the ECM fraction. Over 400 proteins displayed differential expression between experimental groups according to p-value 0.05 and over 100 proteins remained with a fold change ≥ ±2 and an adjusted pvalue of 0.05. Upregulated enriched GO pathways with denosumab treatment included inflammatory response, myeloid cell activation, myeloid leukocyte migration, neutrophil and granulocyte activation (all padj <6.26E-28). Cell morphogenesis was amongst the most downregulated GO pathways (padj 3.42E-23). IPA core analysis of the proteomic dataset showed that the "oxidative phosphorylation" and "tyrosine biosynthesis IV" canonical pathways were significantly upregulated following treatment with Denosumab (padj<1.06E-2). Inflammatory response including activation of macrophages and phagocytes were also found to be upregulated following treatment with Denosumab (padj=1.61E-2).

DISCUSSION: While the ECM enrichment protocol was well suited to unlock the patient material proteome, changes on ECM proteins observed after treatment were minor. These data provide the first human data of the mechanistic effect of denosumab treatment in inflammatory osteolytic lesion activity.

Poster presentation

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Serum proteome profiles in Crohn's disease patients with different phenotypes towards biomarker identification

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Introduction

Crohn"s disease is a chronic inflammation of the gastrointestinal tract leading to progressive, often irreversible bowel damage (strictures, fistulae, functional impairment). The complexity and heterogenous presentation highlights the need for adopting individualised approaches. Biologic therapies are advised particularly in "high risk" patients, to prevent progression. These predictors, however, have been derived from observational, retrospective studies, being unable to reliably distinguish patient subgroups or guide decisions. Proteomics has yielded significant insight into inflammatory bowel disease (IBD), from differentiating subtypes and disease stages to predicting therapy response. However, no biomarker has been implemented, and accurate prognostication is yet to be achieved.

Objective

To analyze the serum proteome of CD patients with different phenotypes by employing mass spectrometry techniques.

Methods

This was a cross-sectional, observational, analytical case-control prospective study. 64 adult CD patients undergoing regular clinic follow-up or hospitalization at a tertiary care center were recruited prospectively between 2016-2018, according to classical diagnosis criteria. CD patients who had either confirmed strictures or penetrating disease at the time of inclusion in this study (n=15), as well as a group of patients with persistent inflammation but no strictures or fistulae (n=15) were selected. Baseline demographic data, disease characteristics and phenotype, as well as the type and duration of IBD treatment were recorded. Blood samples for inflammatory biomarkers, as well as for proteome profiling, and fecal samples for faecal calprotectin measurement were collected during admission. CD patients were followed-up for a period of one to two years to assess disease progression and outcome.

Serum samples collected from the 30 patients and 15 healthy controls were subjected to depletion of highly abundant proteins and to label-free nano-LC coupled with Q-TOF mass spectrometry analysis.

Results

The serum proteome was characterized and over 300 non-redundant proteins were identified in each sample group with good coefficient of variation (CV<15%) and peptide coverage. Proteins that could aid diagnosis, but also disease course and prognosis were identified and comprise potential biomarker panels. A subset of proteins was selected for further results validation using complementary methods.

Conclusion

Global proteome profiling of Crohn"s disease patients with different phenotypes towards biomarker identification was employed by using state-of-the art mass spectrometry techniques. Unique biomarker panels based on protein sets were identified that could aid significant insight into aspects of inflammatory bowel disease (IBD), from differentiating subtypes and stages of the disease to predicting response to therapy.

Poster presentation

P159

Plasma Kynurenine: A Promising Marker for the Assessment of Renal Functions

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Background:

Chronic kidney disease (CKD) represent a worldwide issue due to the high prevalence and the serious complications including death. Accumulating evidences have suggested the association between CKD and the activation of tryptophan metabolism throughout kynurenine pathway, thus the elevation of peripheral kynurenine levels. The alteration in kynurenine levels related to kidney functions and may predict CKD occurrence and complications. The aim of this study was to evaluate kynurenine levels in CKD patients, and to examine the relationship between kynurenine levels and kidney functions indicators (estimated glomerular filtration rate (eGFR), creatinine and urea).

Material and methods: The study included 66 CKD patients in stages 3 to 5 seen at Tishreen University Hospital, and 22 subjects served as control. kynurenine levels were measured by kynurenine ELISA kit (IDK® immundiagnostik).

Results: Kynurenine levels were significantly increased with the increase in CKD stage ($p<0.001$), and it were correlated with eGFR ($r=-0.631$, $p<0.001$), creatinine levels ($r=-0.464$, $p<0.001$), and urea levels ($r=0.528$, $p<0.001$). Kynurenine plasma levels didn"t influenced by age, sex, diabetes and hypertension in CKD patients.

Conclusion: Kynurenine plasma levels may be a promising marker for estimating kidney functions. Furthermore, measuring kynurenine in plasma gives insight into the alteration in tryptophan metabolism in CKD patients, thus may predict the complications in the course of CKD. Key words: chronic kidney disease, estimated glomerular filtration rate, creatinine, kynurenine, tryptophan

P160

Proteomic analysis of *Cupriavidus necator* adapted to various stress conditions

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Cupriavidus necator is a metabolically versatile gram-negative bacterium considered as model organism for polyhydroxyalkanoates (PHAs) metabolism. PHAs are natural, renewable, and biocompatible biopolymers that are accumulated in form of intracellular granules by numerous prokaryotes. These materials serve primarily as carbon and energy storage compounds, nevertheless, according to recent findings, it seems that PHAs play a much more complex role in the stress survival and adaptation of microorganisms to various environments. In this work, during the long-term evolutionary engineering, the wild-type culture of *C. necator* was exposed to selected stress factors (Cu2+, NaCl etc.) to investigate the importance of PHAs in adaptation to selected stressors. According to those results, PHA plays important role in the adaptation of the bacterium to numerous stressors.

Our goal was to compare wild-type strains with evolved cultures also from the perspective of proteomics which can provide a unique broad-spectrum picture on changes associated with adaptation of *C. necator* to stress conditions. We compared 5 conditions in 4 biological replicates. Compared were strains adapted to Cu2+ and NaCl cultivated in presence of these stressors with the wild-type culture cultivated in the presence and even in the absence of the stressors.

The obtained cultures of *C. necator* were lysed in 95°C hot SDT buffer (SDS/DTT/Tris HCl). The protein lysates were processed by the filter-aided sample preparation (FASP) method. Proteins were alkylated, digested by trypsin on the filter unit membrane and resulting peptides were eluted by ammonium bicarbonate. LC-MS/MS analyses of all peptide mixtures were done using RSLCnano system connected to Orbitrap Exploris 480 spectrometer. MS data were acquired in a data-independent strategy.

Acknowledgement CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2018127, is gratefully

acknowledged for the financial support of the measurements at the CEITEC Proteomics Core Facility.

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Investigation of Lysosomal Protein Interactions and Turnover via Cross-Linking and Pulse SILAC Data Independent Acquisition Mass Spectrometry

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Objective: The lysosomal proteome consists of luminal proteins, which are predominantly hydrolases responsible for the degradation of substrates, and membrane proteins fulfilling various functions. These include, but are not limited to, lysosomal acidification, nutrient transport/sensing, and interaction with other proteins, organelles, or complexes. While certain proteins and complexes have been investigated in great detail, the function, structure, half-life, and interaction partners of a significant number of lysosomal proteins have not been analyzed yet. In this project, cross-linking mass spectrometry (XL-MS) in combination with interactive computational modeling and pulsed (p) SILAC data-independent acquisition (DIA) MS under different perturbational cellular states was applied to investigate the lysosomal proteome.

Methods: Lysosomes were enriched by superparamagnetic iron oxide nanoparticles and cross-linked with disuccinimidyl sulfoxide (DSSO). pSILAC labeling was performed for six different conditions and samples taken across seven time points. SCX fractionated XL samples were measured by MS2/MS3 CID/ETD and pSILAC lysosomal fractions by DIA MS, both on an Orbitrap Fusion Lumos. Identified XL interactions were verified and further analyzed via homology-based modeling, co-immunoprecipitation, blue native PAGE, and immunofluorescence microscopy.

Results: We present the first XL and pSILAC DIA MS study of lysosomes. The data contain a highly interconnected network of 847 proteins forming 1024 interactions, including 67% potentially novel interactions, of which two were confirmed by co-immunoprecipitation. Furthermore, our XLs provide evidence for a novel tetrameric assembly of PPT1 and allowed us to propose the first ab initio structures for the monomer, dimer, and oligomeric state of FLOT1/2. Immunoprecipitation of FLOT-positive early endosomes in combination with DIA further revealed the putative cargo of these vesicles. The pSILAC DIA MS study allowed for the determination of 33,968 half-lives for 6,915 unique proteins across wild-type lysosomes and such from cells impaired in: autophagosome-lysosome fusion proteasome activity, cathepsin activity, cholesterol homeostasis, and protein glycosylation. The dataset reveals a median half-life of 18 h for lysosomal proteins in wild-type cells, with differences of up to 30 h for specific proteins in perturbed states. The data imply that lysosomal degradation is the primarily responsible factor for the regulation of protein half-life rather than synthesis rates.

Conclusion: Our data present the first lysosomal cross-linking and turnover dataset, providing a valuable resource for the identification of interactions, analysis of protein structures, and study of lysosomal/proteasomal substrates.

P164

A membrane-permeable and IMAC-enrichable crosslinking reagent for advanced in vivo crosslinking mass spectrometry

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Objective

Crosslinking mass spectrometry (XL-MS) is an attractive method for the proteome-wide characterization of protein structures and interactions. Currently, the depth of in vivo XL-MS studies is more behind the

established applications to cell lysates, because crosslinking reagents that can well penetrate intact cells and strategies to enrich crosslinked peptides still lack in efficiency. To tackle these limitations, we develop a phosphonate-containing crosslinker, tBu-PhoX that efficiently permeates various biological membranes and can be robustly enriched using routine Immobilized Metal Ion Affinity Chromatography(IMAC).

Methods

Cells were harvested and re-suspended in phosphate-buffered saline (PBS) and washed 3 times with PBS. Crosslinking reaction was performed twice, each time with 1 mM tBu-PhoX for 30 min at room temperature. Cells were lysed and cross-linked proteins were digested following a standard protein digestion protocol. The digestion mixture was subjected to a pre-clearance step using IMAC beads to remove endogenous modified (especially phosphorylated) peptides that would interfere with crosslink enrichment. The pre-cleared digestion mixture (i.e., flow through from IMAC) was incubated in dilute trifluoroacetic acid (TFA) solution to remove the t-butyl groups and expose the phosphonic acid group for a secondary IMAC enrichment. Finally, crosslinks were enriched using a standard IMAC procedure and analysed by LC-MS for crosslink identification.

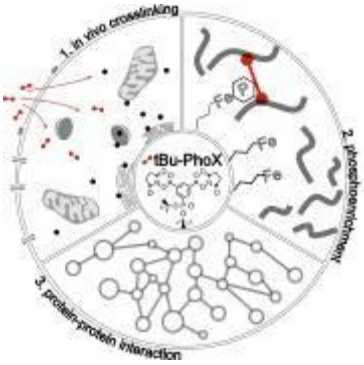
Results

We applied our in vivo XL-MS workflow with optimized parameters to intact HEK293T cells and identified 3,103 crosslinks (6,845 CSMs) in a single-shot 180 min LC-MS measurement. Remarkably, 99.8% of the identifications are phospho-attached (i.e., monolinks, looplinks, and crosslinks), underscoring the highly efficient IMAC enrichment and removal of non-crosslinked peptides. To further improve crosslink identification, we further enriched crosslinks from monolinks by size exclusion chromatography (SEC). Summing up results from six crosslink enriched SEC fractions, we obtained a total number of 9,208 crosslinks from in vivo tBu-PhoX crosslinked HEK293T cells.

Conclusion

We establish a tBu-PhoX based in vivo XL-MS pipeline, achieving high crosslink identification numbers in intact human cells with substantially reduced analysis time (18 hours in our study compared to an average of 240 hours in other proteome-wide XL-MS studies using non-enrichable crosslinkers). This result highlights that tBu-PhoX and our integrated sample preparation pipeline present a highly promising chemical approach for advancing in vivo interactomics and structural biology.

Fig. 1



P166

Mass spectrometry (Interactome analyses, Top-Down MS, and HDX-MS): Swiss Army knife to dissect profound structural and functional rearrangements of proteasome complexes 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, thymoproteasome t20S). The proteolytic activity is regulated by different proteins and protein complexes, such as the 19S, PA28α/β, PA28γ, PA200, and it has been shown that these activators would bind preferentially to certain proteasome types [1]. A new subunit has been discovered recently, called α4s; it 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 [2]. KO models of the PA200 and PA28γ regulators are also infertile, which makes them interesting candidates for potential functional partners of s20S in spermatogenesis.

Knowing which proteins sptP interacts with and deciphering the nature of these interactions could help understand why is α4s so crucial for spermatogenesis.

Methods: We approached this question in two different ways: 1- exploring the dynamics of proteasome composition and interacting proteins throughout spermatogenesis 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) that we have recently implemented on the proteasome in the lab [3].

Results: After analyzing the germ cells at different stages of development, we obtained a differential image of the proteasome composition and of its interaction partners. 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 (98%) to predominantly s20S (>82-92%) 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.

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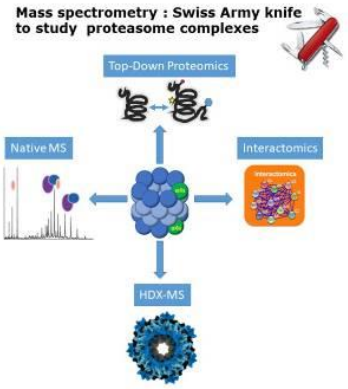
Fig. 1



P167

A Protein-Nucleic Acid Cross-Linking Node for Proteome Discoverer 2.5

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Introduction: Advances in cross-linking mass spectrometry of protein-DNA and protein-RNA complexes have recently emerged, but data visualization and processing remain a key challenge. The OpenMS cross-linking search engine NuXL (manuscript in preparation) significantly improves data processing and cross-link detection between proteins and RNA/DNA moieties. Proteome Discoverer 2.5 (PD) offers interactive visualization and node-based data processing capabilities. Making the novel NuXL tool available inside the PD graphical user interface (GUI) makes state-of-the-art computational methods for XL discovery easy to configure, execute and visualize.

Methods: We developed a novel plugin compatible with PD 2.5 that provides custom nodes for nucleic-acid cross-link analysis with result visualization. The plugin is written in C# and wraps the configuration and execution of NuXL as well as parsing and processing of the produced cross-linking results.

Results: After installation of the NuXL PD nodes, the user can conveniently configure the tool through the PD GUI, execute the search and visualize results. Presets for several cross-linking protocols are readily available and can be selected by users to match their experimental setup. After execution, the XL-FDR level filtered cross-link spectrum matches are made available for inspection in a result table. Manual inspection of cross-link spectrum matches is possible though our custom extension to PD that allows visualizing cross-link spectra along with extensive fragment annotations provided by the NuXL search engine.

Access to the binary installer for the PD node is available upon request and subject to a beta testing agreement.

Conclusions: Integration of the OpenMS NuXL nucleic acid search engine as Proteome Discoverer 2.5 node significantly lowers the barrier for researchers to perform data analysis in the emerging field of protein-RNA/DNA cross-linking.

P168

Inspection and service on mitochondrial complexes ensure bioenergetic function in postmitotic tissues

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Investigations on cell lines offer only limited information about the dynamics of mitochondrial protein complexes in differentiated tissues. **Rationale:** It is an open question how the functions of mitochondrial complexes are maintained in postmitotic cells. **Objective:** We aimed to investigate turnover of proteins to understand if OXPHOS complexes undergo quality check and repair mechanisms. **Method:** Complexome profiling (CP) combines blue native electrophoresis with quantitative mass spectrometry and identifies components of protein complexes and scarce assembly intermediates. A combination of CP and pulsed SILAC allowed the identification of protein exchange within complexes. **Results:** We collected comprehensive data of turnover and half-life of single proteins in mitochondrial macromolecular complexes. A complete remolding of OXPHOS complexes required about a month. Moreover, we explored the maintenance plan of complex I and identified a time-dependent assembly of freshly translated proteins into spare parts and exchange of old modules. We applied this technique to study mitochondrial disorders and discovered novel factors involved in inspection and service of complex I. **Conclusion:** We showed that repair mechanisms on protein complexes ensure full bioenergetic function in differentiated tissues.

P169

Activation of the prothrombin and complement systems appears to be involved at a different level in the progression of colorectal cancer

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Question

Colorectal cancer (CRC) is one of the most common cancers. Early detection of CRC remains a challenge and the identification of new biomarkers represents a relevant target. Several authors highlighted that serum peptidomic patterns may be correlated with events occurring in all districts of the organism. Mass spectrometry is well suited for the detection of low molecular weight proteins that might well represent a dynamic reflection of tissue function. The aim of the present work is the determination of a serum peptidome signature able to help in the CRC diagnosis and prognosis.

Methods

Blood samples from 180 patients with CRC and 70 healthy volunteers were collected. The extraction of the low molecular weight fraction of serum proteome has been performed using Dynabeads RPC 18 beads.

MALDI/TOF spectra were acquired in a range from 800 to 3000 m/z. All serum samples were tested for their integrity by means of Seradeg (1), a publicly available web tool aimed at assessing the quality of sera. According to this analysis, only a subset of 170 samples was used in the following comparison. The final set included 170 samples, of which 92 patients had been diagnosed with metastatic CRC, 78 patients had been diagnosed with non-metastatic CRC, and 20 were healthy volunteers. The lists of significant peaks were analysed by means of Geena 2 (2), a publicly available web tool for pre-processing of MALDI/TOF. The output generated by Geena 2 was then analysed by means of SAMR (3), in order to identify signals significantly different in the main data sets, i.e. healthy volunteers, metastatic CRC and non-metastatic CRC. The findings were also validated by a bootstrap resampling procedure. Multiple regression models were used to validate the findings of the SAM analysis taking into account the differences in age and gender between patients and controls.

Results

The SAMR analysis put in evidence a peptide signature able to distinguish between healthy volunteers and CRC patients, which includes the m/z 1561.72 (human prothrombin P00734, fragment "TATSEYQTFFNPR") and m/z 2021.08 (C3 complement P01024, C3f complement fragment "SSKITHRIHWESASLLR"). In particular, our results show that the intensity of these two signals was higher in the serum samples from CRC patients than in those from healthy controls. Interestingly, while the difference for m/z 2021.08 signal was only significant between healthy volunteers and metastatic CRC, the difference for m/z 1561.72 was significant between healthy volunteers and non-metastatic CRC too.

Conclusions

Our results showed that prothrombin and complement system activation are involved in CRC progression. However, they may provide a novel approach for a better classification of CRC patients only after a validation on an independent larger set of sample.

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P170

Mass spec-based detection of protein alterations in male breast cancer serum and tissue

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Background/Aim: Male breast cancer (MBC) is a scarce entity; it accounts for less than 1% of all malignancies in men. Thus far, data on MBC are extrapolated from studies based on its female counterpart, owning to the rarity of the disease. Additionally, treatment modalities are prescribed in a categorical manner, due to the lack of individualized biomarkers focusing on male patients. Despite highlighting this unmet need, the protein expression profile of male breast cancer tissue and serum remains understudied. On the one hand, serum represents an attractive, non-invasive source for biomarker discovery. On the other hand, tissue-based biomarkers are used routinely to guide therapy selection. In the current study, we employed mass spectrometry (MS)-based proteomics to identify a panel of proteins that are differentially regulated in male breast tumors and pre-operative serum clinical samples. **Materials and Methods:** Two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) were employed to detect differences in serum protein expression between eleven patients with MBC and healthy controls. Additionally, the protein content of four male breast tumors and corresponding normal adjacent tissues was analyzed by high-throughput nano-liquid chromatography-MS/MS (HPLC-MS/MS) technology. **Results**: A panel of differentially expressed serum proteins between patients with MBC and healthy individuals was identified. Specifically, analysis of the resulting 2-DE gels revealed 504 protein spots differentially expressed between the two groups; two hundred and fifty-two

proteins with diverse biological roles were identified since more than one spot was related to the same protein. Furthermore, 2352 proteins were detected in malignant tissue samples, that correspond to 1249 single gene products. Of those, a panel of 119 differentially expressed tissue proteins was identified in MBC samples compared to normal controls; 90 were found to be overexpressed in MBC tissues, whereas 29 were downregulated. Concomitantly, 844 proteins were detected only in MBC tumors and 197 were expressed exclusively in normal mammary samples. **Conclusions**: Our results demonstrate altered protein expression in malignant tissue and serum, leading to increased understanding of MBC pathogenesis and contributing towards the identification of more accurate markers that have the potential to be clinically meaningful.

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Compared dia-PASEF and prm-PASEF approaches for the absolute quantification of 500 human plasma proteins in colon cancer plasma samples

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Introduction

Targeted proteomics methods are a traditional choice for absolute protein quantitation of proteins in biofluids, as they allow to obtain a high selectivity, specificity and sensitivity while resolving the missing value problem for a limited number of targets. We recently evaluated the prm-PASEF acquisition strategy which allows to further increase the number of addressable targets and the method"s selectivity without compromising the sensitivity. In parallel, if the dia-PASEF approaches now widely used to address the missing value problem in discovery studies have theoretically a reduced specificity, they have however a greater multiplexing potential, we are now applying both approaches 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 25cm X 75µm pulled emitter column (IonOpticks, Australia) packed with 1,6µm particles 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 1564 precursor-ions corresponding to 782 peptides from 565 proteins while using a 2 min retention time window. The median relative standard deviation of the signal of the peptides was of 3%. We demonstrated accuracy over more than 3 orders of magnitude of peptides concentrations with a maximum error on the determination of 20%. 98% of the 574 quantified peptide pairs could be quantified from the prm-PASEF experiment, while 96% could be quantified from the dia-PASEF experiment. The results obtained by both approaches were highly correlated. The limit of quantitation obtained from the prm-PASEF experiment was down to 7.4 amol on column whereas it was down to 20 amol on column with dia-PASEF.

Conclusions

Both approaches have been successfully applied to the analysis of colon cancer plasma samples and allowed to spot regulated proteins that had formerly been spotted from cancer tissue analysis.

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Multiomics examination of serum and tear from patients with obesity or type 2 diabetes

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Objective

Diabetes mellitus is one of the most common metabolic diseases. Being obese for a prolonged time may lead to permanently high glucose concentration in the bloodstream and subsequently the development of type 2 diabetes in obese individuals. Patients with type 2 diabetes may face many complications, especially at high risk of retinopathy triggered by diabetes. Therefore, our main objective was to analyze proteins and metabolites, including amino acids and biogenic amines, in serum and tear samples from patients with type 2 diabetes, patients with obesity, and a matched control group.

Methods

A total of 85 subjects were recruited for this study, 26 patients with type 2 diabetes, 31 obese subjects, and 28 healthy volunteers. 100 µl of a serum sample, 3 µl of tear sample from each participant were analyzed. The concentration of 20 proteinogenic amino acids, 3 non-proteinogenic amino acids including taurine, citrulline, and ornithine, and 10 biogenic amines: histamine, ethanolamine, methylamine, ethylamine, putrescine, serotonin, cadaverine, tyramine, and phenethylamine, were determined using AccQ-Tag Ultra derivatization (Waters), and UPLC (Waters) coupled to 5500 QTRAP (Sciex) mass spectrometer. The proteomes were analyzed using Easy nLC 1200-Orbitrap Fusion (ThermoScientific) system following trypsin and Lys C digestion.

Results

All the examined compounds have been detected. Serum concentrations of Ser, Gly, Asp, Glu, Thr, and Cit were significantly decreased, while serum Cys, Val, Ile, and Leu levels were increased in both disease groups compared to the control group. The concentrations of serum Cys and Cit were statistically significantly higher, whereas serum levels of Asp and Ile were lower in the obese group compared to the type 2 diabetes group. All amino acids were quantified in tear samples, but only Arg level was significantly increased in the type 2 diabetes group compared to the obese group. Compared to serum, the level of amino acids was lower in tear, however, the level of Ser, Asp, Trp, and Cit was similar in the two sample types. Only serum ethanolamine was significantly decreased in the disease groups compared with the control group. Methylamine, ethylamine, putrescine, and serotonin were detected in serum but not quantified. In tears, 9 biogenic amines were detected, but only ethanolamine was quantified. The protein lists acquired from the proteomics examination of serum and tear samples were examined along with the data acquired from the examination of small molecules.

Conclusion

Our results may enlighten the metabolic alterations related to obesity and type 2 diabetes and may provide a better understanding of these complex diseases.

This research was funded by GINOP-2.3.3-15-2016-00020, NKFI FK134605, and Tempus Public Foundation - Stipendium Hungaricum Scholarship 2019.

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Data-independent proteomic analysis of hyperoxia induced attenuation of migration in cultured oligodendrocyte progenitor cells.

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Objective: Cerebral oxygenation disturbances, like hypoxia–ischemia and premature exposure to high oxygen levels, contribute to the pathogenesis of brain lesions in preterm infants with white matter damage. These children are at risk of developing long-term neurodevelopmental disabilities. Post injury, extensive migration of oligodendrocyte precursor cells (OPCs) to damaged axonal tracts is required for efficient remyelination. However, a clear understanding of the molecular mechanisms underlying oxidative stress affecting these important physiological processes in the brain is still lacking

Poster presentation

Methods: We investigated the effect of hyperoxia (80% O2) on the protein composition and migration capabilities of immature oligodendrocytes (OLs) using the OLN93 (rat-derived OPC) cell line as an experimental model. After a 24-hour exposure to hyperoxia, we monitored the changes in the proteome profile of the cells by data-independent acquisition on a QExactive HFx mass spectrometer. The migration abilities were analysed by a wound healing assay. Functional categorization of proteomic results we carried out using Ingenuity Pathway Analysis. Particularly important pathways were analysed in detail by immunoblotting.

Results: Proteomic analysis of five independent bioreplicates covered 3612 unique protein groups and revealed significant changes of 431 proteins between cells cultured under normoxic and hyperoxic conditions, respectively. Functional assignment showed enrichment of proteins in pathways regulating cytoskeletal remodelling, cell migration, and cell survival. Especially, actin cytoskeleton signalling, signalling by Rho Family GTPases, ephrin receptor signalling and Rac signalling were seen to be inactivated. Furthermore, a strong activation of Nrf2 mediated oxidative stress response pathway was observed along with RhoGDI signalling. In line with this, hyperoxia led to impaired migration of the OLN93 cells in culture. The observed effects could be related to changes in levels of the cofilin/LIMK pathway associated proteins. Hence, higher levels of phosphorylated cofilin and phosphorylated LIM kinase upon hyperoxia point to an increase of inactive cofilin, whereas higher values of TESK and chronophin support tight regulation of the inactive and active cofilin portions.

Conclusion: The study disclosed an impaired migration of OPCs upon hyperoxia and the associated molecular alterations on protein level. We predict the pathways mentioned above to be the upstream active effectors that might finally lead to the attenuation of migration under hyperoxic conditions and might be used therapeutically, as targets to combat oxidative stress induced brain damage in preterm infants.

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Examination of salivary biomarkers for identification of oral malignancies using DIA spectral libraries

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Objective: Oral squamous cell carcinoma (OSCC) has the 6th highest prevalence among the malignant diseases worldwide. Besides the genetic determination, the main etiological risk factors are smoking and alcohol consumption. The 5-year survival rate of OSCC is only 50% therefore, the early diagnosis is critical for the appropriate treatment. Precancerous stages of OSCC are oral leukoplakia (OLK) and oral lichen planus (OLP). OLK is defined as a white plaque, which cannot be scraped, used as an exclusionary diagnosis. Its prevalence is 2,6% worldwide and the chance of malignant transformation is approximately 25%. OLP is an immune mediated chronic inflammatory disease, which affects the mucous membrane of the oral cavity. The frequency of transformation to cancer is approximately 5%. The gold standard methodology of the diagnosis is based on biopsy and histological examination but it cannot be used for screening. Examination of salivary biomarkers is a promising new screening tool. Our aim was to identify salivary protein biomarkers specific for OLK, OLP and OSCC with mass spectrometry analysis.

Methods: Saliva samples of patients suffering from OLK, OLP and OSCC and of age- and sex-matched controls were collected from four different Hungarian clinics. Protein concentration was determined using Bradford and BCA method and the samples were digested with trypsin. The digested saliva samples were analyzed using data-independent acquisition (DIA) mass spectrometry.

Results: DIA spectral libraries were generated by the analysis of OLK, OLP and OSCC groups and the generated libraries were used for screening for new potential salivary biomarkers.

Conclusion: With the developed DIA library, the proteomic analysis of salivary samples were possible,

and found to be a useful screening method for new salivary biomarkers for OSCC and its precancerous states.

Funding: *This research was funded by GINOP-2.3.3-15-2016-00020. We would like to thank the help of the MSS Lab Ltd.*

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Anti-AGO1 antibodies identify a treatment-responsive subgroup of sensory neuronopathy: diagnostic and prognostic biomarker detected via two complementary proteomic methods

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The presence of specific autoantibody (Ab) biomarkers in neurological diseases assists clinicians to establish a diagnosis and treatment decisions. However, for many patients, no biomarker is detected, sometimes despite suspicion of an autoimmune context. To reveal a potentially undetected autoimmune context, novel Abs are of major importance, as the treatment of neurological diseases with an autoimmune context differs from those without. In this study we aimed to identify a novel serum Ab biomarker of an autoimmune context in neurological diseases.

We applied two complementary proteomics methods for two disease cohorts concerning the peripheral and central nervous system. In a first approach, the serum of a limbic encephalitis patient with an uncommon immunohistochemical fluorescence pattern and a control serum without such pattern where used for immunoprecipitation of their targeted antigens. After in-gel digestion, peptides were analyzed via nanoLC-MS/MS. In a second approach, Human Proteome (HuProt) protein microarrays, comprising 16,000 distinct bait proteins were applied for antibody identification in 12 patients with sensory neuronopathy (SNN) with a suspicion of an autoimmune context, 22 disease controls with other neuropathies, and 9 healthy controls. Enzyme-linked immunosorbent assay (ELISA) was used to validate the Abs in larger cohorts and to determine Ab titer and IgG subclass.

Via both proteomic approaches, we identified Abs against the family of Argonaute (AGO) proteins (mainly AGO1 + AGO2) as autoantibody candidates. Via ELISA, we screened the sera of 823 subjects for AGO1 Abs: 433 patients with peripheral neuropathies, 274 with systemic autoimmune diseases (AID) already known to be associated with AGO Abs as a positive control cohort, 116 healthy controls (HC) as a negative control. Among peripheral neuropathy patients, 28/433 (6.5%) had AGO1 Abs. In SNN patients (17/132 [12.9%]), the frequency was significantly higher than in non-SNN neuropathies (11/301 [3.7%]; p=0.001), AID (16/274 [5.8%]; p=0.02), and HC (0/116 [0%]; p<0.0001). AGO1 Abs titers ranged from 100-100,000. IgG subclass was mainly IgG1. Regarding the clinical pattern, AGO1 Abs-positive SNN patients were more severe than AGO1 Abs-negative ones (SNN score: 12.2 vs. 11.0, p = 0.004; face affected: 8/17 [47%] vs. 14/75 [19%], p = 0.01; global areflexia: 13/17 [76%] vs. 29/75 [39%], p = 0.01). AGO1 Abs-positive SNN showed more frequently a positive response to immunomodulatory treatment than AGO1 Abs-negative SNN (7/13 (54%) vs. 6/37 (16%), p = 0.02). Multivariate logistic regression adjusted for potential confounders showed that response to treatment was only associated with the presence of anti-AGO1 Abs (OR 4.93, p = 0.03).

Our findings suggest a diagnostic and prognostic benefit of AGO1 Abs as a novel and early biomarker for autoimmune-related SNN. A similar benefit in other AGO1 Abs-positive diseases (e.g., encephalitis) should be addressed in future studies.

Poster presentation

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Dissecting phospholamban (PLN) p.Arg14del cardiomyopathies on the proteome level

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Objective

Phospholamban (PLN) p.Arg14del cardiomyopathies are genetically encoded heart diseases characterized by superinhibition of the sarcoendoplasmic reticulum Ca^{2+} pump, frequently presenting as lethal arrhythmias and progressive cardiac dysfunction. While the underlying genetic mutation shows regional prevalence in the Netherlands, it is widespread globally. The regulatory function of PLN-WT on SERCA2a-mediated Ca^{2+} transport is significantly increased by phosphorylation at S16 and T17, whereas Arg14del abolishes S16 phosphorylation by removing the PKA-C recognition motif and thus reduces SERCA2a stress regulation. We investigated the effect of the Arg14del mutation on functional interactions of PLN with key proteins in Ca^{2+} homeostasis, as well as on the global proteome and phosphoproteome.

Methods

V5-APEX2-PLN overexpressing Neonatal rat cardiomyocytes (NRCMs) were used for proximity proteomics. Isolated adult mouse cardiomyocyte lysates provided total membrane preparations from WT and heterozygous p.Arg14del mice; cardiac membrane preparations from human patient samples were generated as described in [2]. Following tryptic bottom-up sample preparation, samples were analyzed by either data-dependent analysis (DDA) mass spectrometry on hybrid quadrupole/time-of-flight instruments, or by data-independent analysis (DIA) on a hybrid ion mobility/quadrupole/time-of-flight instrument.

Results

(i) APEX2 proximity labeling analysis in NRCMs pointed to 14-3-3 phosphoadaptor proteins binding to PLN in its physiologically relevant pentameric (pPLN) state, thereby blocking PP1c phosphatase access to S16/T17 and keeping SERCA2a in the high-activity pump state. Vice versa, S16 dephosphorylation reduces 14-3-3/pPLN binding and thus promotes monomeric PLN which inhibits SERCA [1].

(ii) BN-PAGE Complexome Profiling in mice identified a novel Ca^{2+} cycling PLN-RyR2-SERCA2a supercomplex with Protein Phosphatase 1 regulatory subunit (PPP1R3A) (Fig. 1). The PLN-RyR2-SERCA2a supercomplex was confirmed in healthy human control samples, however was disrupted in paroxysmal and chronic atrial fibrillation patients [2]. This effect was tentatively confirmed in heterozygous p.Arg14del mice compared to wildtype.

(iii) DIA mass spectrometric profiling of p.Arg14del and wildtype mice shows significant downstream effects on the cardiac proteome, specifically on proteins of the OxPhos system and the inner mitochondrial membrane. Phosphoproteome studies are ongoing and will be presented.

Conclusion

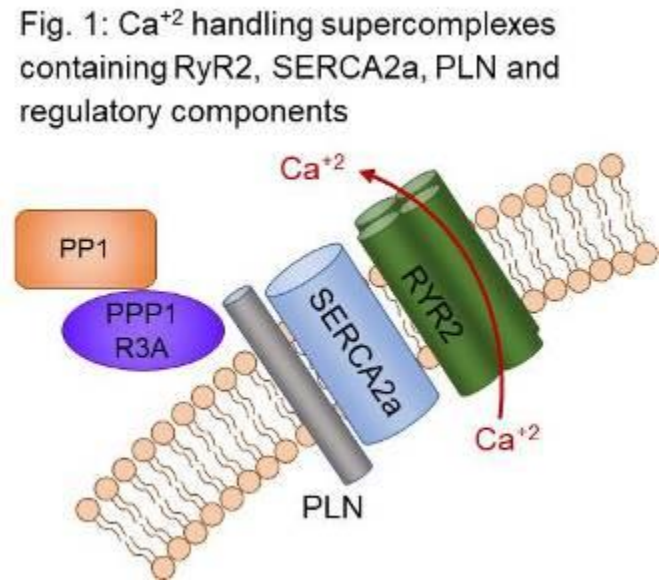
Mass spectrometric proteome analysis reveals a significant impact of the PLN p.Arg14del mutation, both on the level of functional protein/protein interactions relevant to Ca2+ homeostasis and on global cardiac energy metabolism. Further studies will increase our understanding of the underlying disease mechanisms and may point to novel therapeutic targets.

[1] Menzel J et al., Science Signaling 13, eaaz1436 (2020).

[2] Alsina KM et al., Circulation 140, 681 (2019).

Poster presentation

Fig. 1



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AltProt, broadening the vision of the protein landscape

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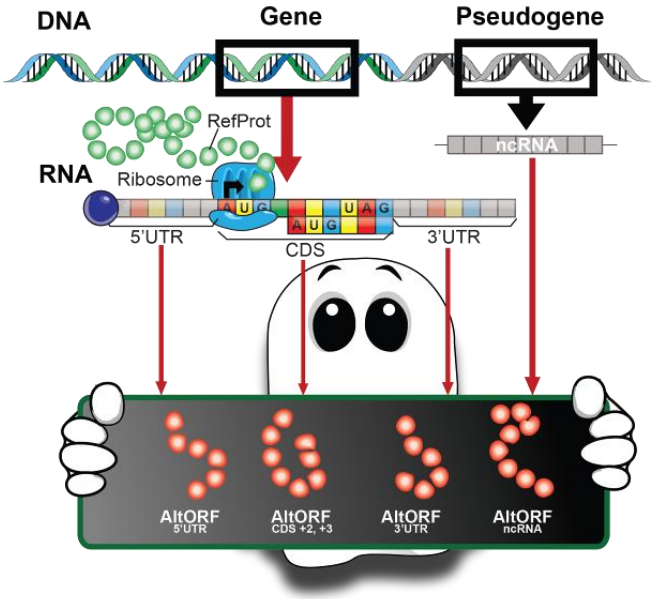
It was conventionally admitted that eukaryotes mature messenger ribonucleic acids (mRNAs) are monocistronic leading to the translation of a single protein. However, large-scale proteomics has led to a massive identification of proteins from alternative open reading frames (AltORFs) translated from mRNAs at the 3"&5" UTR or by a shift of the CDS (+1 or +2) in addition to the predicted proteins issued from the reference open reading frame (RefORF) but also recovered from non-coding RNAs (ncRNAs). These alternative proteins (AltProts) are not represented in the conventional protein databases and this "Ghost proteome" was never considered until recently. However, these proteins were shown to be functional and there are growing evidences that they are involved in central functions such as cellular regulation both in the physiological and physiopathological context. Ghost proteins, therefore, represent a novel world, filling the gap in the understanding of signalling pathways, establishing as new markers of pathologies and therapeutic targets.

To open the vision of the proteomic landscape, I proposed a large description of our studies, mainly focused on biomarker research in cancer. I propose to address the findings made in clinical and research studies on the presence of several AltProt in breast cancer, and in particular certain observation of membrane AltProt maybe the next generation target for CAR therapy. In the pathology of Glioma, a large number of ncRNAs present in their sequences an ORF allowing their translations into AltProt. AltProts have been shown to be implicated in protein-protein interaction (PPI) with reference proteins (RefProt) reflecting involvement in signaling pathways linked to cellular mobility and transfer RNA regulation. More recently, clinical studies have revealed that AltProt are also involved in the patient"s survival and bad prognosis. A presentation of our observation in ovarian cancer will also be approached on the basis of the differential study of 3 cancer cell lines also involving a study of PPIs. Finally, I propose to present the presence of AltProt involved in the pathology of COVID-19, partners of viral proteins as well as potential unreferenced expression of viral RNA.

Fig. 1



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Elucidating the role of alternative proteins (AltProts) in ovarian cancer by studying protein networks

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Eukaryotic mRNA has long been considered monocistronic, by which a single reference protein (RefProt) is translated from a reference open reading frame (ORF). Large-scale (MS-based) proteomics relies on databases (DBs) for peptide and protein identification. It is often observed that a fraction of good quality MS/MS spectra does not match to sequences stored in DBs. Amongst others, this challenges the concept of monocistronic mRNA, hence new DBs such as OpenProt, predict proteins translated from alternative ORFs (5'&3'-UTR, lncRNAs, or in-frameshift), with their proteins named AltProts. As the identification of AltProts is now possible, their functions are still undeciphered and here, interactomics can add by mapping such proteins to biological pathways. Large-scale crosslinking-MS (XL-MS) is an attractive technique to identify protein-protein interactions (PPIs) of AltProts. Moreover, such strategies can be complemented by targeted approaches like BioID and Virotrap. This study aims to identify AltProts in Ovarian Cancer (OvCa) models and to decipher their functions in the pathology.

OvCa cell lines (PEO-4 & SKOV-3) and an immortalized ovarian cell line were analyzed to identify their RefProts and AltProts. Likewise, the quantitative variation of proteins was also assessed. In total, 5,045 RefProt and 453 AltProt were identified by combining two proteome extraction methods (RIPA and SDS). Among them, 34 AltProt were found using both extraction methods, implying that both extractions are complementary. Using PCA, AltProts show a clear clustering between healthy and cancer cells as well as, between the two kinds of cancer cell lines, these results are consistent with those obtained with RefProts. Moreover, a significant variation of AltProt (313) and RefProt (2,894) levels in cancer and healthy cells was observed. In these cell lines, PPIs were analyzed using the DSSO crosslinker, without crosslinked peptide enrichment, but using the subcellular fractionation of a restricted number of cells (3E6) whereas one typically uses huge numbers of cells (300E6). Correlation with the shotgun proteome analysis confirmed variable proteins recovered in the XL-MS network. The resulting network was expanded using STRING and GO term information, providing the first insights into the physiological involvement of AltProts.

In conclusion, AltProts are a neglected part of the human proteome which might play a role in OvCa. Moreover, proteogenomics can be used for their identification due to the advances of NGS and analysis algorithms. The first approach to unravel its biological function is XL-MS. However, the development and improvement of XL-MS techniques are crucial. Particularly, decreasing the amount and complexity of the sample analyzed without the use of enrichment techniques. Afterward, their roles have to be validated by other techniques like for instance BioID, Virotrap, development of an antibody, and over-expression.

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In-depth phosphoproteome analysis of anti-EGFR therapy secondary resistant colon cancer derived from PDX model

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Objective: With more than 1.9 million cases worldwide in the year 2020 alone, colorectal cancer (CRC) is the third most commonly occurring type of cancer. Currently, targeted anti-epidermal growth factor receptor (anti-EGFR) therapy is one of two available treatment options leading to improved survival in stage IV CRC. Unfortunately, the vast majority of patients that originally was therapy-sensitive, develops a secondary resistance over the course of the treatment. To elucidate the underlying molecular mechanisms, a panel of anti-EGFR therapy secondary resistant patient-derived xenograft (PDX) tumors was developed. In this model, primary tumors from therapy-naïve CRC patients were implanted into immunocompromised mice, called avatars. The tumors

received targeted anti-EGFR treatment in the avatars until they progressed and acquired a secondary resistance. Tumors were harvested in untreated, treated and secondary resistant states. As the molecular alterations between those states become manifest as changes in cellular signalling, which in turn is mediated by phosphorylation, our objective was to develop a workflow for in-depth phosphoproteome analysis of PDX tumors.

Methods: We analyzed each specimen in technical quadruplets to account for intra-tumor heterogeneity as well as generating robust and reliable data on the individual sets of sensitive, treated, and secondary resistant tumors. After lysis and tryptic digest, the samples were labeled with tandem mass tags (TMT) in order to maximize the precision of the relative quantification. The samples then were pooled and fractionated using high pH reversed phase chromatography to reduce the complexity and to maximize the depth of analysis. The phosphopeptides of the resulting 12 subfractions were enriched using TiO_2 affinity chromatography and were subsequently analyzed via synchronous precursor selection (SPS) MS^3.

Results: On average, more than 11,000 phosphosites were identified. Of those, around 8,000 could be quantified in at least three of four replicates with a site probability of over 75 % and a signal-to-noise ratio of more than 10:1 allowing for a highly accurate in-depth view of the steady state phosphorylation patterns of the different tumors. Data analysis revealed over 1,700 phosphosites to be differentially expressed between the secondary resistant tumors and the tumors being treated with anti-EGFR therapy.

Conclusion: Overall, the approach shows great promise to give valuable insights into the development of secondary resistance. In future experiments more sets of tumors will be analyzed. The tumor-specific mechanisms of secondary resistance will be investigated and compared between individual PDX models. Eventually, significantly differentially phosphorylated sites might be verified and further studied using immunological methods and targeted mass spectrometry to elucidate their functional role in the development of secondary resistance.

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Quantitation using stable isotope labelled peptides to set up a diagnostic protein panel in the differentiation of renal carcinoma and normal kidney tissue

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Objective: By *in silico* methods using gene array and RNA-Seq datasets from clear cell Renal Cell Carcinoma (ccRCC) patients with paired normal tissue samples a set of genes overexpressing in ccRCC were revealed. Evaluation of protein-level changes related to these genes was performed to assemble a panel of proteins with diagnostic value in the differentiation of normal and tumor kidney tissue.

Methods: First, tryptic digestion of test kidney samples (normal and tumor) was performed to select peptide candidates of target proteins for stable isotope labelled (SIL) peptide generation. Then SIL peptide based quantitation of the selected proteins from paired control (tissue surrounding tumor) and tumor samples was performed to reveal protein-level changes related to the overexpressed genes in ccRCC.

Results: Only 21 out of the 30 targeted proteins were detected even after high pH fractionation of the tryptic digest of normal and tumor tissue test samples. For the rest, data from Protein Explorer/Massive were used to select 1-5 peptides per protein for SIL peptide synthesis. In total, 75 SIL peptides were monitored in 81 normal and tumor tissue sample pairs. Finally, 22 proteins were quantified from 44 target peptides, of which 18 proteins showed significant changes: the amount of 17 proteins increased and 1 protein decreased in the tumor region compared to the surrounding normal tissue.

Conclusion: Using SureQuant method significant changes also at the protein level were confirmed for about 3/5 of target genes with overexpression in renal tumor tissues, of which a support vector machine-based algorithm assembled a minimal set of proteins that can potentially be used in the diagnosis of renal carcinoma.

Poster presentation

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Glycomic and proteomic characterization of liver tissues with cirrhosis and hepatocarcinoma

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Objective: The aim of our work was to perform a glycomics (chondroitin sulfate, CS, and heparan sulfate, HS) and quantitative proteomics study of samples from hepatocarcinoma (HCC), cirrhotic and cancer adjacent cirrhotic human liver parenchyma tissues with various etiologies, such as Hepatitis B and C infections (HBV and HCV), primary sclerotizing cholangitis (PSC), and alcoholic liver disease (ALD).

Methods: Formalin-fixed paraffin-embedded (FFPE) human liver tissues were investigated. Glycosaminoglycan (GAG) chains were degraded into disaccharides by on-tissue bacterial lyase digestion, followed by graphite+C18 TopTip purification. Samples were analyzed using nanoHPLC-MS/MS with self-packed HILIC-WAX capillary column. Proteins were degraded into peptides by on-tissue tryptic digestion, followed by Pierce C18 purification. Analysis of samples was performed using reversed-phase nanoHPLC-MS/MS. Proteins were identified by Byonic and compared between sample groups by Scaffold. The label-free quantification was performed with MaxQuant. The protein-protein interaction network was created with STRING GO webserver. Statistical analysis was performed with R Studio.

Results: Regarding total intensities of CS and HS chains, strong etiology dependence was observed between HBV- and HCV-associated cirrhotic tissues; while CS was more prevalent in HBV-associated cirrhosis, HS level were higher in HCV-associated cirrhosis. It was also observed that ALD-associated HCC resulted in ca. 3-times lower CS levels than other HCCs.

Examining the sulfation patterns of CS, a significant difference in 6-*O*-sulfation was observed between cirrhotic tissues with different etiologies, and no etiology-dependence was shown for HCC. Regarding HS, PSC-associated cirrhosis showed decreased sulfation, but no other etiology-dependence was observed. However, a shift in *N*-sulfation and *O*-sulfation was observed between cirrhosis and HCC samples. Comparing monosulfated disaccharides, the *N/O*-sulfated ratio was 1.9 times higher in HCC, and for doubly sulfated disaccharides this change of ratio was 3.4.

During the proteomic studies, we performed qualitative and quantitative analysis of cirrhotic samples. We identified an average of 1340 proteins from each sample. During the label-free quantitation of proteins, we found 38 proteins with significant changes. No proteoglycans showed significant difference.

Conclusion: We observed differences in the total amount and sulfation pattern of CS and HS disaccharides in correlation with etiology of cirrhosis and HCC. These changes, however, were not governed by proteoglycan expression.

Acknowledgements: The research was carried out with the support of the National Talent Program (NTP-NFTÖ-20-B-0203). Lilla Turiák acknowledges the support of the National Research, Development and Innovation Office (OTKA PD 121187) and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

Poster presentation

Fig. 1

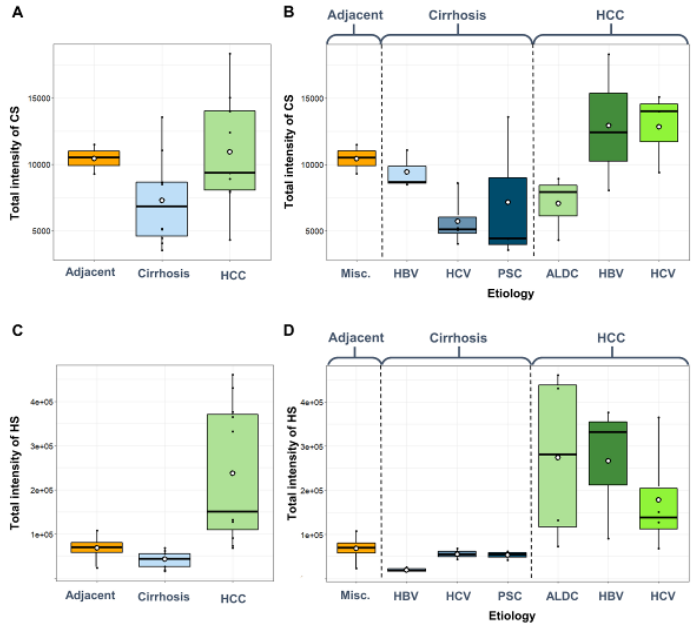
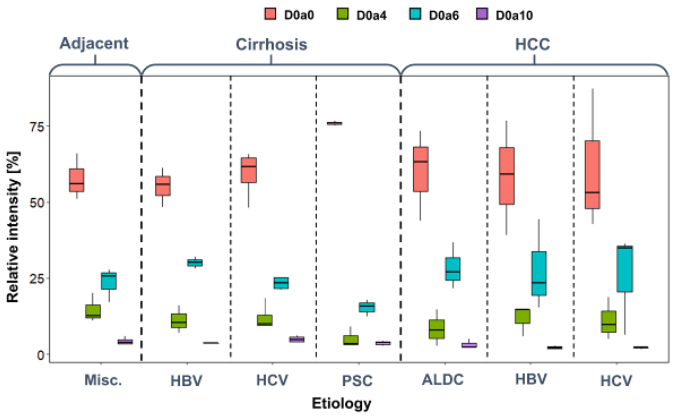


Fig. 2



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Proteomic mapping of severe anthracycline cardiotoxicity phenotype by conventional liquid chromatography coupled to high-resolution mass spectrometry

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Question: Despite their effectiveness as anticancer drugs, anthracyclines (e.g. doxorubicin and daunorubicin) may induce extensive toxic damage of the heart leading to chronic cardiomyopathy and heart failure. Exploratory proteomic analysis can provide a valuable and complex insight into molecular changes underlying development of cardiotoxicity. With implementation of "high-res" mass spectrometers and modern chromatographic columns, there is a growing interest to utilize conventional (high-flow) liquid chromatography also for bottom-up proteomic analysis. However, there is still a question if conventional LC-MS/MS analysis is able to cover and explain global protein changes associated with severe anthracycline cardiotoxicity phenotype in such scale as far more traditious nanoLC-MS/MS.

Methods: Three samples of homogenized rabbit myocardium representing a severe anthracycline cardiotoxicity phenotype (daunorubicin – DAU, 3mg/kg, weekly 10 weeks) were compared to the control myocardium samples (saline administered to rabbits in the same time schedule). All six samples were processed by protein enzymatic digestion followed by Tandem Mass Tag isobaric peptide labelling. To cover a rabbit myocardial proteome on a maximum scale, two-dimensional (2D) peptide separation was realized using sample fractionation in basic pH followed by separation of collected fractions on reversed C18 phase. By this approach, 72 fractions were collected in total and pooled in final 24 fraction analysed by conventional liquid chromatography coupled to high-resolution mass spectrometry (LC-MS/MS).

Results: More than 4000 proteins were quantified using conventional LC-MS/MS. Subsequent quantitative data evaluation and comparative analysis revealed that severe anthracycline cardiotoxicity phenotype was associated with marked global protein dysregulation; significant up- and downregulation with fold change ≥ 2 were found in 385 proteins and 463 proteins, respectively. Observed protein dysregulation consisted of profound remodelling of cardiomyocyte sarcomere and other structural proteins (e.g., myosin heavy and light chains, troponins, myomesin and desmin) as well as components of extracellular matrix (e.g. fibronectin and periostin). Pro-inflammatory proteins, proteasome subunits and multiple antioxidant proteins were markedly up-regulated, whereas the levels of mitochondrial proteins like voltage dependent anion channels, subunits of respiratory chain and superoxide dismutase (SOD2) were declined compared to control myocardium.

Conclusions: Conventional LC-MS/MS is a versatile proteomic tool for comprehensive mapping of

proteome changes related to the development of severe anthracycline cardiotoxicity phenotype and having a great potential to become full-fledged alternative to nanoLC-MS/MS.

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Multiplexed Apolipoprotein Profiling Advances the Assessment of Residual Lipid-Related Cardiovascular Risk

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Objective

Low-density lipoprotein cholesterol (LDL-C) is among the conventional lipid parameters used to predict risk of cardiovascular disease (CVD). Statins lower blood levels of pro-atherogenic LDL-C, but a residual cardiovascular risk remains in some individuals with therapeutically optimised LDL-C levels. Although the metabolism of LDL-C and other lipoprotein particles is governed by a range of different apolipoproteins, only apolipoproteins A-I and B are measured in clinical assays. Using a more comprehensive apolipoprotein panel in a large epidemiological cohort, this study aimed to determine the association of individual apolipoprotein levels with risk of coronary heart disease (CHD).

Methods

Bottom-up multiple reaction monitoring–mass spectrometry (MRM–MS) was used in conjunction with stable isotope-labelled peptide standards to quantify plasma levels of 13 apolipoproteins in participants of the Precocious Coronary Artery Disease (PROCARDIS) study (N = 1916; 941 cases of CHD, 975 controls). The relationship between apolipoprotein levels and CHD was assessed after adjusting for established risk factors for CVD and correcting for statin use.

Results

The strongest positive associations with CHD in the PROCARDIS study were seen for triglyceride-related apolipoproteins C-I (odds ratio [OR] 2.38, 95% confidence interval [CI] 1.63–3.46), C-III (OR 2.95, 95% CI 1.85–4.71) and E (OR 2.35, 95% CI 1.54–3.58), as well as for apolipoprotein (a) (kringle IV type 2 repeat, OR 2.84, 95% CI 2.04–3.95). Comparing these with associations of apolipoproteins with CVD in the Bruneck study (N = 688) revealed consistency across the two cohorts. Robust inverse associations with CHD were observed for apolipoproteins A-IV (OR 0.45, 95% CI 0.31–0.65) and M (OR 0.29, 95% CI 0.19–0.44).

Conclusion

Analysing two large epidemiological cohorts, Bruneck and PROCARDIS, demonstrated that multiplexed apolipoprotein profiling improves the understanding of cardiovascular risk independent of conventional lipid parameters. Most prominently, triglyceride-related apolipoproteins were shown to positively associate with residual cardiovascular risk. The findings of this study support the need for development and implementation of standardised, MRM–MS-based apolipoprotein profiling assays to guide novel lipid-modifying therapies beyond statins.

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High-Coverage Proteome Analysis Using 2D Conventional-Flow LC-MS

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Objective

Efficient peptide separation via liquid chromatography (LC) represents an essential prerequisite to maximize protein sequence coverage by mass spectrometry (MS) and uncover proteome changes reflecting pathological conditions, especially, in case of higher number of samples. Isobaric labeling of peptides, e.g. Tandem Mass Tag (TMT), enables time-effective sample multiplexing but the accuracy of protein quantification can be underestimated compared to label-free approach (LFQ). Moreover, both approaches can be hardly affected by instability of electrospray during long LC gradient working at flow rate lower than 1 µL/min (nanoLC). Here, we present an optimization of 2D TMT conventional-flow LC-MS (68 µL/min) with aim to assess its viability and effectiveness compared to nano LC-MS used as a benchmark.

Poster presentation

Methods

In this study, we utilized three distinct model samples: i.) lysate of mouse B lymphoblasts, ii.) lysate of mouse B lymphoblasts spiked with *S. pneumoniae* lysate and iii.) homogenized and lysed rabbit myocardium. Proteins in all samples were digested to peptides by trypsin and analyzed using LFQ approach or derivatized by TMT isobaric labels. Protein coverage and quantification accuracy achieved by 2D conventional-flow (68 µL/min) LC-MS were discussed in comparison with nanoLC-MS (250 nL/min) and 1D-LC peptide separation. Moreover, optimization of conventional-flow LC-MS was accompanied with several MS parameters adjustment, e.g. dynamic exclusion, resolution in MS2 mode, isolation window or collision energy.

Results

TMT-based approach was in our hands slightly less accurate than LFQ, as shown on mixed species proteome model, but balanced by significant instrument time-reduction. However, first trial of TMT-labeled samples using conventional-flow LC-MS ended up with very low number of quantified proteins. Due to systematic optimization and switch from 1D to 2D LC, the number of quantified proteins in TMT-labeled myocardial samples increased from 276 to 4023. For example, 2D approach with 24 concatenated fractions instead of 8 and with shorter gradient time increased number of quantified proteins by 30% at the same overall gradient time. We thus achieved only 9.5% of quantified proteins less than using 2D nano LC-MS approach. Moreover, we did not observe any decrease of quantified proteins between replicates using conventional-flow LC-MS. In contrast, number of quantified proteins in 2nd technical replicate dropped by 7.7% in the same time period (3 days) using nano LC-MS.

Conclusion

We optimized 2D conventional-flow LC-MS analysis to get high proteome coverage and to get close to 2D nano LC-MS in number of quantified proteins. Deeper proteome characterization using 2D workflow was achieved at the expense of increased MS time, where the robustness and stability of conventional-flow LC approach was beneficial.

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Proteomic investigation of cortico-limbic brain tissues from chronic social stress (CSS) mice provides insights into the aetio-pathophysiology of major depressive disorder (MDD)

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Objective: According to the World Health Organization, major depressive disorder (MDD) is a leading cause of global disability affecting approximately 121 million people worldwide. However, the understanding of the aetio-pathophysiology is limited, and no MDD biomarker profile is available for diagnosis, prognosis, and assessment of treatment response. A mouse model for MDD-relevant psychopathologies based on chronic social stress (CSS) includes dysregulation of the prefrontal cortex (PFC) and amygdala (AMY). This study constituted a proteomic investigation of PFC and AMY in CSS mice, thereby providing translational data of potential relevance to the aetio-pathophysiology of MDD.

Methods: For 15 days, male C57BL/6J mice underwent control handling (CON, N = 12) or chronic social stress (CSS, N = 15). The latter comprised co-housing with dominant CD-1 mice separated by perforated Plexiglas and daily placement in the same compartment for 10 min or a cumulative attack time of 60 s. After sacrifice on day 16, proteins were extracted from PFC and AMY microdissections, digested on carboxylated magnetic particles and eluted in three fractions by decreasing isopropanol concentrations. The fractions were analyzed by LC-MS/MS utilizing 180 min gradients, 3 compensation voltages (CV) alternating every 1s and data-dependent acquisition. Raw data were separated

by CV and analyzed using MaxQuant 2.0.3. Database search was performed against the UniProt mouse reference proteome, and label-free quantification was enabled for relative quantification.

Results: In total, 122,000 peptides in 7500 proteins were identified. Of these, more than 5400 proteins were quantified in at least 65% of samples for one condition and brain region. Distinct CSS effects on protein levels were observed in both brain regions. More than twice as many proteins were affected in the AMY (498) than in the PFC (186). In PFC, one of the proteins most down-regulated in CSS mice was cell cycle exit and neuronal differentiation protein 1 (Cend1). A point mutation in human Cend1 has been identified as a risk factor for increased depressive symptoms. In AMY, the two proteins most up-regulated in CSS mice were hyaluronan and proteoglycan link protein 2 (Hapln2) and inter-alpha-trypsin inhibitor heavy chain H3 (Itih3); both are linked to the organization of hyaluronic acid-mediated processes in the extracellular matrix, especially neuronal conduction. Enrichment analysis for biological processes affected by CSS showed that in PFC essentially no processes were enriched, and in AMY the processes regulation of synaptic transmission and plasticity were enriched.

Conclusion: This study provides a comprehensive dataset of proteins affected in the prefrontal cortex and the amygdala in mice exposed to CSS, a manipulation that leads to MDD-relevant behavioral states. The results provide potentially valuable translational insights at the proteome level into the aetio-pathophysiology of MDD.

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Comparative proteome analysis of tegument of male and female adult *Schistosoma mansoni*

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Objective: Schistosomiasis affects about 240 million people worldwide, causing approximately 200,000 deaths annually. Causative pathogens are parasitic blood flukes that can survive for decades in their mammalian final host. The surface of male and female *Schistosoma* spp. allows them to survive successfully in their hostile environment without being attacked by immune defense. Here we present the first gender-specific tegument proteome datasets of paired and virgin adult *Schistosoma mansoni*.

Methods: We applied a new workflow, where a streptavidin-biotin affinity purification technique was combined with single pot solid-phase enhanced sample preparation (SP3) for subsequent LC-MS/MS analysis.

Results: We identified 1519 tegument proteins for female and male worms out of a pair and female and male worms originated from single-sex infection. Bioinformatic analysis revealed that proteins involved in signaling pathways of cellular processes and antioxidant mechanisms were exclusively found among the female-specific tegument proteins, while the male-specific proteins were enriched for those involved in protein phosphorylation processes and signal transduction.

Conclusion: Our results indicate that adult *Schistosoma mansoni* have sex-specific tegument proteins that may explain the induction of several immune responses in the host. This knowledge has to be considered to find a holistic solution to this global health problem of schistosomiasis.

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MS^n ion mobility-mass spectrometry data visualization and exploration with mineXpert2

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Mass spectrometry has the peculiarity of being a technique almost universally applicable to any kind of analyte. Depending on the nature of the analytes under scrutiny, the mass data generated by the instrument are going to vary a lot from an application to another, like structural biology, proteomics or metabolomics, for example. In order to visualize and

explore mass spectrometric data, there is, however, a common set of data processing requirements that any useful software should implement. We have developed the *mineXpert2* software with usability in mind. *mineXpert2* eases the full-depth visualization and exploration of mass spectrometry data at any arbitrary MS^n level. Ion mobility-mass spectrometry (IM-MS) data are fully supported and a variety of data handling processes are tailored to these specific data exploration needs.

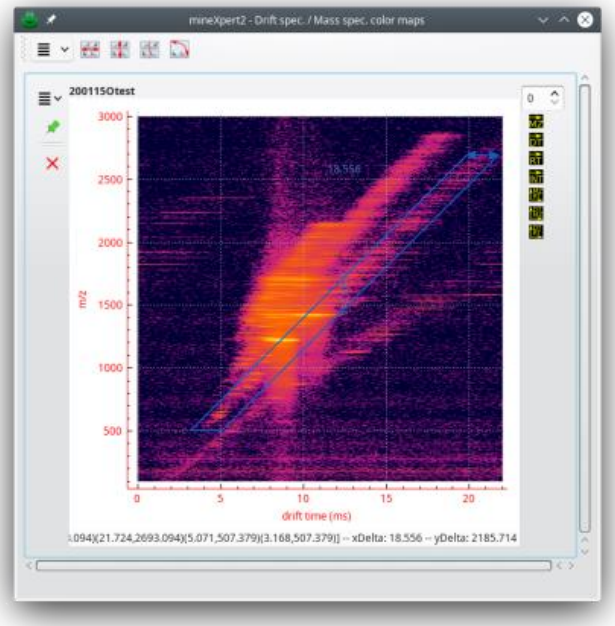
The power of *mineXpert2* comes from its ability to perform a variety of mass spectral data integrations so as to explore the data along different orthogonal paths, like integrating data from a total ion current (TIC) chromatogram to a drift spectrum or integrating data from a drift spectrum to a mass spectrum, for example. Color maps are available to display retention time *vs* mass spectrum, retention time *vs* drift spectrum and retention time *vs* mass spectrum data relations. It is possible to indefinitely chain integrations along any path, effectively reducing, step-by-step, the data to be displayed so as to pinpoint the tiniest mass spectral feature of interest. For example, once a color map has been computed and displayed, it is possible to reverse-integrate data from it to a TIC chromatogram, a mass or a drift spectrum. *mineXpert2* is geared towards human-eyed mass spectrometric data comparison and interpretation, particularly by allowing one to load any number of mass spectra, to process them and to visualize and compare them in novel ergonomic and flexible ways. This is achieved by providing the user with original data plotting features that allow the most intuitive data comparison process.

From an internal mass data handling perspective, *mineXpert2* allows the user to finely configure the data processing, like m/z binning. This feature makes *mineXpert2* a universal program capable of coping with a huge variety of mass spectral data storage specifications by the different vendors. An advanced system to record to file the user analysis findings allows one to perform decentralized annotation of mass spectrometric data.

mineXpert2 reads mass spectrometry data files in the mzML, mzXML, MGF, txt formats. It is written in portable C++11 for the three major computing platforms, with full parallelization of all the mass data integration code, *mineXpert2* has seen its performance vastly improved over the previous version.

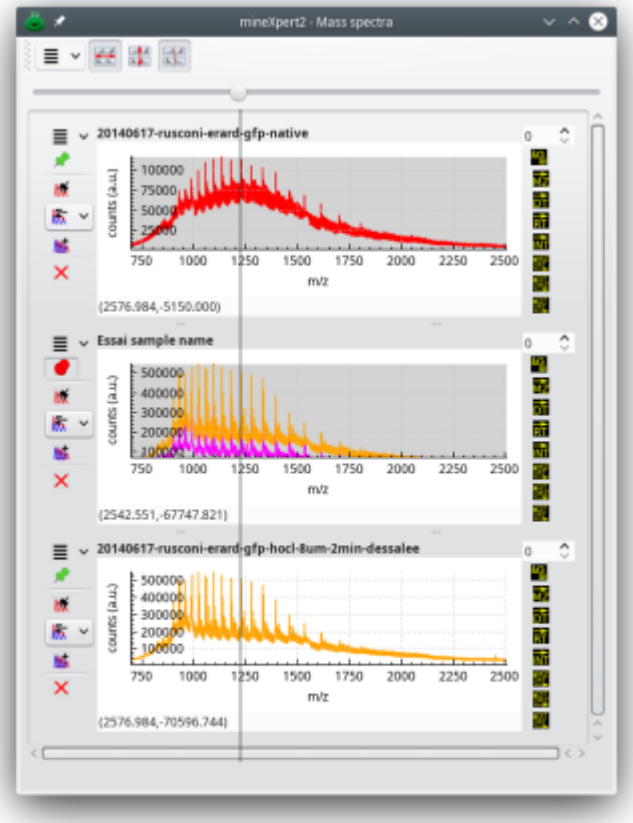
mineXpert2 is Free Open Source Software, needing no proprietary software whatsoever and is freely downloadable at http://www.msxpertsuite.org (no request needed) along with a highly detailed user manual.

Fig. 1



Skewed rectangle selection from a m/z vs drift spectrum color map

Fig. 2



Multiple MS data files display
Each plot widget has a set of features associated to it.
Integrations are selected using the right hand side buttons.

P188

The genome of the single human chromosome as a gold standard for its transcriptome

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One of the key steps in transcriptome profiling is to determine the criteria for uncovering gene expression; that is, to establish the appropriate threshold for identifying whether or not a gene is expressed. The cutoff level applied in sequencing analysis varies according to the sequencing technology, sample type, and study purpose, which can largely affect the coverage and reliability of the data obtained. However, there is a need for a "gold standard" transcriptome data analysis, which would enable obtaining complete transcriptome coverage of the genome of interest, such as that encoded by a single chromosome. In this study, we sought to establish such a gold standard using human chromosome 18 (Chr 18) as an example, aimed to determine the optimal combination of parameters for reliable RNA transcriptome data analysis.

Toward this end, we compared the results obtained from different transcriptome analysis platforms (quantitative polymerase chain reaction, Illumina RNASeq, and Oxford Nanopore Technologies MinION) for the transcriptome encoded by human chromosome 18 (Chr 18) using the same sample types (HepG2 cells and liver tissue). A total of 275 protein-coding genes encoded by Chr 18 was taken as the gene set for evaluation. The combination of Illumina RNASeq and MinION nanopore technologies enabled the detection of at least one transcript for each protein-coding gene encoded by Chr 18. This combination also reduced the probability of false-positive detection of low-copy transcripts due to the simultaneous confirmation of the presence of a transcript by the two fundamentally different technologies: short reads essential for reliable detection (Illumina RNASeq) and long-read sequencing data (MinION). The combination of these technologies achieved complete coverage of all 275 protein-coding genes on Chr 18, identifying transcripts with non-zero expression levels. This approach can improve distinguishing the biological and technical reasons for the absence of mRNA detection for a given gene in transcriptomics.

This work was supported by the Russian Science Foundation (RSF Grant 20-15-00410). The authors are grateful to the "Human Proteome" Core Facility, Institute of Biomedical Chemistry (IBMC) for performing data processing.

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Uniquome: Deciphering the human uniquome

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Unique Peptide (UP) was previously defined as the amino acid sequence appearing solely in one protein and split in two categories; **Core Unique Peptides** (CrUP) and **Composite Unique Peptides** (CmUP), which together comprise the **Uniquome** (Figure 1).

Poster presentation

In this study, we present the human Uniquome. Except from the recording of Unique Peptides we analyzed some special characteristics of them such as the amino acid size and starting position of Unique Peptides. Furthermore, their coverage and density in the Human proteome was calculated. In order to create a new approach in protein identification by Mass spectrometry with the use of these peptides, we searched in the tryptic digest peptides if they contain unique peptides, and we recorded the tryptic digest unique peptides. Finally, in order to understand the physiological role of unique peptides, protein families, and other Peptide Databases like the cancer protein database and the immunopeptide database were searched for the existence of Unique Peptides.

20,430 reviewed human proteins (Uniprot Database) were analyzed and the analysis showed that the Human Uniquome consist of 7.263.888 Core Unique Peptidess, and 77.697 Composite Unique Peptides which means 64% and 0,68% density of UPs, respectively (Figure 2). From the total of the reviewed proteins, only 148 proteins were found to contain no Unique peptides. Further examination of the identified Unique Peptides showed that the majority of Core Unique Peptidess and Composite Unique Peptides are comprised of 6 AA and 11 AA, respectively. Position analysis revealed that the Core Unique Peptides appeared with the same ratio in the proteins and the vast number of Composite Unique Peptidess were identified in the beginning of the proteins. Comparative analysis of cancer antigenic and immune epitope peptides for Core Unique Peptides and Composite Unique Peptides indicated that 89% of cancer antigenic and 87% immune epitope peptides included in these databases consisted at least by 1 Unique Peptide. Finally, the analysis for tryptic digest unique peptides showed that the 99% of Human reviewed proteins contain at least one tryptic digest unique peptide.

Uniquome can be very useful in the identification of proteins by mass spectrometry and the application of selected reactions monitoring complex protein mixtures. Furthermore, the Uniquome studies across species will unearth new knowledge on protein functions, while orchestrating novel strategies for drug discovery, antibody design and disease therapy.

Fig. 1

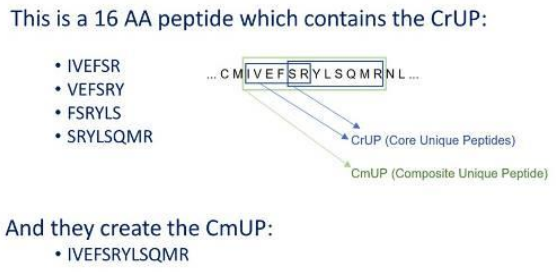

Fig. 2

Human Uniquome	
Proteins (reviewed)	20.430
Proteins with Unique Peptides	20.282
Proteins without Unique Peptides	148
Core Unique Peptides	7.263.888
Composite Unique Peptides	77.697
Density of Uniquome	64%
Coverage by Uniquome	93%

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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, despite a quadrupoles fidelity is not sufficient to separate MOMA peptides by their *m/z*. This is especially valuable for PTM analysis, for example to resolve positional isomers of phosphopeptides. TIMS Viz was introduced to the real-time database search engine PaSER to visualize MOMA events in complex samples and was herein used to analyze data sets obtained from a whole cell lysate and phosphopeptide enriched sample.

Poster presentation

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), operating in PASEF acquisition mode. An Aurora Series UHPLC column (25 cm x 75 µm, 1.6 µm C18, IonOpticks) was used for chromatographic separation. Data analysis was performed using the real-time database search engine PaSER (Bruker Daltonics). The novel TIMS Viz tool was used for data visualization in form of a precursor heatmap in *m/z* and ion mobility dimensions and for identification of MOMA features.

Results

During acquisition, the MS/MS spectra are streamed to a GPU-powered processing computer performing a real-time database search, called PaSER. The database search utilizes all four dimensions – retention time, CCS value, *m/z* and fragment spectra – to increase confidence in the identification 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 in two different data sets with different *m/z* tolerance settings. Setting tolerances to 500 mDa (typical lower limits of quadrupole isolation) 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. 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.

Conclusion

TIMS Viz, a software tool was introduced for data visualization to explore mobility offset mass aligned (MOMA) features in m/z and ion mobility dimension

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Characterization of peptide-protein relationships in protein ambiguity groups via bipartite graphs

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Objective

In bottom-up proteomics, proteins are enzymatically digested before measurement with mass spectrometry (MS). Because of this, peptides are identified and quantified directly from the MS measurements and the inference and quantification of proteins from this peptide-level data remains a challenge. The relationship between proteins and their corresponding peptides can be represented by bipartite graphs. The aim of this study is to characterize and structure the different types of graphs that occur and to compare them between data sets. Furthermore, we want to show how this knowledge can aid relative protein quantification, especially for proteins without unique peptides.

Methods

We construct bipartite peptide-protein graphs using quantified peptides from two measured data sets, as well as theoretical peptides from an in silico digestion of the corresponding protein sequence databases. Then, the graphs are grouped into isomorphism classes (sets of graphs with the same structure, see figure). The results are compared between data sets as well as between database and quantitative level. We also apply a new relative protein quantification method to the quantitative peptide-level data, that makes use of the bipartite graph structure.

Results

We observed a large influence of the minimum peptide length used during the in silico digestion. The inclusion of very small peptides leads to the formation of an

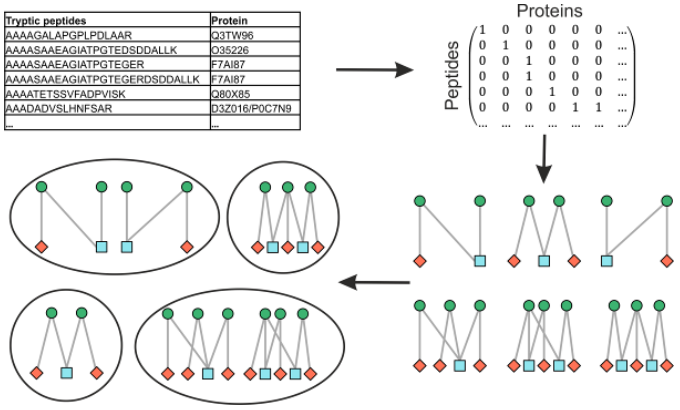
extremely large graph where most proteins are connected via chains of shared peptides. The change of the graphs from the theoretical peptides to the measured ones is subject to two opposite effects. On the one hand, the graphs based on measured peptides are on average smaller and less complex compared to graphs using all theoretically possible peptides. On the other hand, the proportion of protein nodes without unique peptides, which are a complicated case for inference and quantification, is considerably larger for measured data. Additionally, also the proportion of graphs containing at least one protein node without unique peptides rises, when going from database to quantitative level.

Conclusion

Large differences between the structures of bipartite peptide-protein graphs have been observed between database and quantitative level as well as between the two analyzed species. In the two analyzed measured data sets, the proportion of protein nodes without unique peptides were 46.6 and 6.4 %, respectively. Especially for these proteins, the usage of information from the bipartite graph structures for relative protein quantification is benefical.

Figure 1: Construction of bipartite peptide-protein graphs and grouping into isomorphism classes.

Fig. 1



P192

Interspecific differences in translational processes in amphipod species from the Lake Baikal region in Siberia upon exposure to temperature stress: Results from proteome and transcriptome comparison analyses

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[5]*Leipzig University, Institute of Biochemistry, Leipzig, Germany*

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Objective: The water of Lake Baikal is highly pristine and cold (mean temperature: 6°C). The lake is inhabited by endemic gammarid species, such as *Eulimogammarus verrucosus* and *Eulimnogammarus cyaneus*. *Gammarus lacustris* is an amphipod species occurring in fresh waters in the Baikal region but it is not found in the lake itself. The three species have different thermal limits and preferred temperatures. Lethal temperatures for 50% (LT50-24 h) of individuals range from 25.2 (*E. verrucosus*) to 27.2°C (*G. lacustris*), reflecting the different temperature sensitivities of the species. To get mechanistic insights into temperature stress-induced changes in amphipods, we compared proteome and transcriptome responses of three non-model amphipod species whose genomes are not yet sequenced upon challenge of individuals with temperature stress. We aimed to investigate how transcriptome and proteome responses relate and which variants of transcripts are expressed as proteins in those species to improve the understanding of their physiological responses to temperature stress. **Methods:** In order to obtain proteome data we performed LC-MS/MS with tandem mass tag (TMT) labeling. We identified candidate coding regions within transcript sequences from RNA sequencing data and used those as basis for proteome assembly of the obtained mass-spectrometry data. Since genome data are not available, we overlapped the assembled

Poster presentation

protein groups with transcript names and compared their log fold changes obtained after differential expression and abundance analyses. **Results:** We examined proteomes of animals exposed to 24°C for 24 h and compared the proteome with transcriptome data of individuals subjected to an identical treatment and of individuals from a 3 h heat shock exposure. In contrast to the other, more thermotolerant, species, the 24 h heat shock proteome of the thermosensitive *E. verrucosus* showed a better correlation with the 3 h heat shock transcriptome than with the 24 h heat shock transcriptome, indicating a drop in the metabolic rates at the more prolonged heat shock exposure (Fig.1). We also examined changes in proteomes in response to heat shock, considering changes in transcripts, and found three response patterns on the transcript and protein levels: 1. matching up/downregulation of a transcript and corresponding protein; 2. unchanged transcript level and upregulated protein; 3. unchanged transcript level and downregulated protein. Differentially abundant proteins were associated with the following cellular processes in all three amphipod species in response to temperature stress: proteostasis maintenance, antioxidant response, oxygen transport intensification, cell adhesion maintenance, and protein synthesis regulation (Fig.2). **Conclusion:** The degree of correlation of transcriptome and proteome appeared to depend on different thermal tolerances of the three examined amphipod species.

Fig. 1

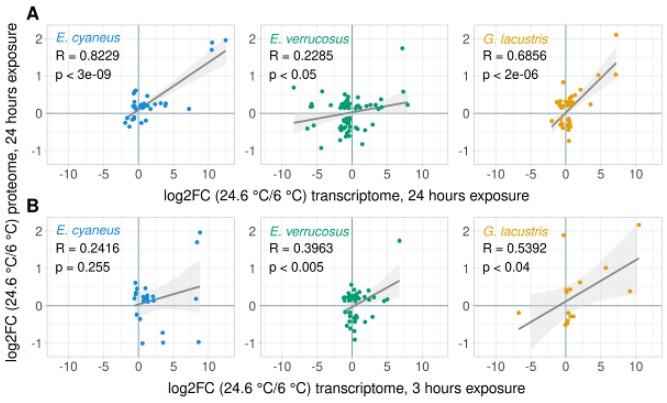
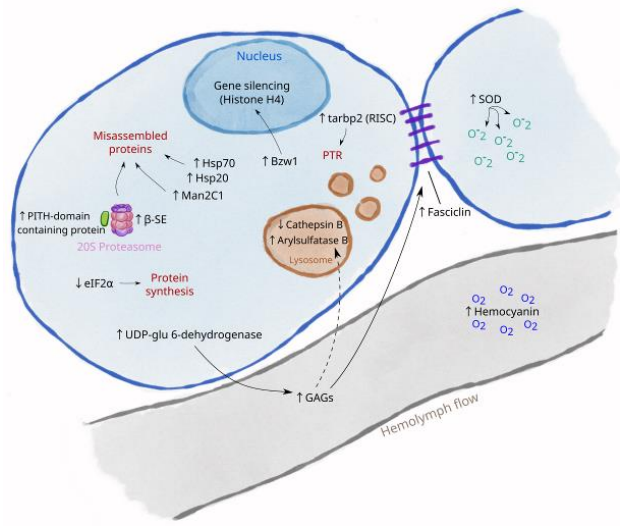


Fig. 2



P193

Pout2Prot: an advanced protein grouping tool

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Objective

Protein grouping is a commonly used strategy to deal with the problem of protein inference. Protein inference is based on peptides identified during a shotgun proteomics experiment, which can be assigned to multiple proteins. It is crucial for cases were multiple species – and therefore homologous proteins – are expected, in particular in metaproteomics. The goal of protein grouping in metaproteomics is to assign proteins to groups in such a way that the taxonomy and protein function assigned reflects best the microbial community of the sample. The objective of this work was to evaluate various protein grouping algorithms in light of these requirements.

Methods

Poster presentation

Four categories of protein grouping algorithms are typically used: (i) The parsimony rule applied (Occam"s razor) or (ii) the parsimony rule not applied (anti-Occam"s razor). Peptides grouped based on (iii) a single shared peptide (groups) or (iv) a complete set of shared peptides (subgroups). In order to evaluate protein grouping tools, we defined 14 test cases that should result in a certain grouping for each of the four categories. The test cases are designed to detect undesired behavior such as: the false application of the parsimony principle (i.e. removing proteins with unique peptides) or assignment of different protein groups depending on the order of processing.

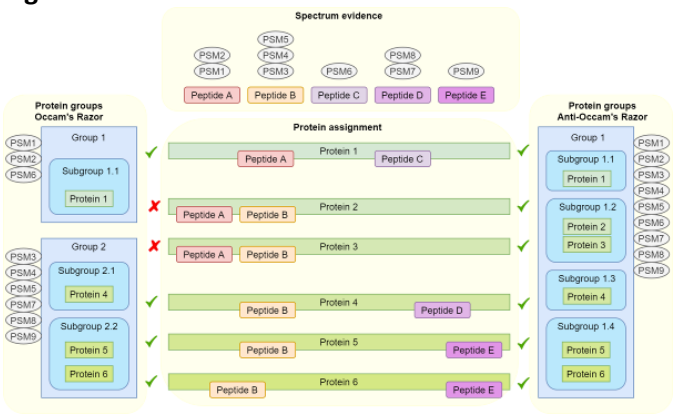
Results

A comparison of four existing protein grouping algorithms applied in FIDO (integrated into Percolator), PIA, X!TandemPipeline and MetaProteomeAnalyzer resulted in various inconsistencies. For instance, PIA will remove a random protein dependent on the order of the input data when applying the parsimony principle. To overcome problems identified with the four methods described above, a fifth algorithm – pout2prot – was developed and included in the comparison. Pout2prot comprised algorithms of all four methods and does not lead to inconsistencies in any of the 14 test cases. Finally, we evaluated how inconsistent grouping can lead to inconsistent assignment of taxonomic and functional annotations in metaproteomics and reflected on how to properly apply protein grouping in the future.

Conclusion

Assessment of protein grouping algorithms demonstrated that all four existing grouping tools analyzed can fail in certain simple test cases leading to inconsistencies. The new algorithm used in pout2prot avoids these problems and we encourage other developers to include it in future versions of their tools.

Fig. 1



P194

Proteomics analysis followed by network analysis reveals proteins and functions characteristic to atheroma and complicated lesions in human atherosclerosis

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Objectives: Atheroma is basically the build-up of macrophages, lipids, and debris of other deposits in the artery wall being the major cause for the narrowing of arteries. Risk factors of atheroma formation include high blood pressure, smoking, diabetes, obesity, and insulin resistance. In order to get more information on the protein composition of atheroma, we aimed to examine the protein composition of atheroma and complicated lesions and to compare their content to healthy artery. The major objectives of our project are to do a comprehensive network analysis of the data originating from the examination of different sample types and to study the post-translational modifications of the proteins with high emphasis on Cys oxidation.

Methods: Proteins from atheromatous lesions, complicated lesions and healthy arteries were

extracted and digested with trypsin. The protein identification was done based on MS/MS spectra with the help of MaxQuant and EncyclopeDIA. The list of identified proteins in each sample was subjected to protein-protein interaction analysis. The String-db was used for network creation and the Cytoscape for network analysis. The ClueGo plugin was used for the pathway analysis, while the gene-gene interactions were retrieved with CluePedia. The top 20 hub proteins were obtained by the administration of cytoHubba. The enriched GO pathways were studied with DAVID as well.

Results: We observed more oxidation events in complicated lesions compared to the other sample types. The oxidations affected mostly but not exclusively the haemoglobin. The functional analysis showed considerable overlaps among the networks. The differentially enriched functions in complicated lesions were the redox processes – being in accordance with the observed increase in oxidation events – as well as receptor-mediated endocytosis, and in atheroma the complement activation and the negative regulation of endopeptidase activity, while in the healthy arteries, the proteolysis.

Conclusion: The workflow used for the deep examination of these sample types can reveal important information which in turn can be used as potential therapeutic targets for clinicians.

Funding: This project is funded by *GINOP*-2.3.3-15-2016-00020 and the Stipendium Hungaricum scholarship.

P195

Trace-back proteome interaction network building of enzymes and regulatory proteins in altered amino acid metabolic pathways in Type 2 Diabetes and Obesity

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Objective

To decipher the underlying changes in the amino acid (AA) metabolism of patients with type 2 diabetes (T2D) and obesity (Ob) based on AA level variations, we applied a systems biology approach by building the interaction network of metabolic pathway enzymes, regulatory proteins and transporters of the AAs of interest.

Methods

Our primary data composed of AAs and biogenic amines (BAs) whose concentrations altered significantly in the serum of patients with T2D or Ob as compared to controls determined by UPLC-coupled mass spectrometry. We retrieved all enzymes and other protein coding genes involved in the metabolic pathways of these AAs and BAs from BioCyc and KEGG databases via their application programming interfaces. After selecting the enzymes directly involved in the formation or degradation of the AAs and BAs, we added the corresponding transporters to create the input query list for STRING network analysis. From experiments and databases as active interaction sources up to fifty of the first shell interactors were chosen at 0.9 confidence level. Taking the generated network, we did further processing and analysis with the "igraph" and "ggraph" R packages, and Cytoscape by including the AAs and BAs as additional nodes and reaction types (synthesis or degradation) as directed edges.

We also performed a gene set enrichment analysis with the "topGO" R package using custom gene ontology (GO) term annotation provided as gene-to-GOs mappings. The map file was generated by an in-house-written shell script that extracts annotation data from daily updated files available on the NCBI FTP site. For the gene set enrichment analysis we defined two sets of proteins: 1) enzymes reacting with the AAs and their transporters as the primary set that directly influence their serum concentrations, and 2) the rest of the pathway proteins with the above mentioned first shell interactors as the background set. As a comparison, we repeated the gene set enrichment analysis with ClueGO, Cytoscape's plugin software.

Results

The enriched functions characteristic for networks generated using data acquired by the analysis of patients with Ob or T2D were very similar. In line with data published in the scintific literature the involvement of Ser, Gly, Glu and Val, Leu, Ile were observed. The sulfur-containing compound metabolism, along with the implication of the retrograde transport was shown to be important in both pathological conditions, and condition-specific functions could also be identified.

Conclusion

The applied workflow and the generated network was appropriate for the complex examination of metabolite levels and we could depict biologically relevant information from the analysis of protein-protein interaction networks of proteins modulating the level of the examined metabolites.

Funding

This research was funded by NKFI FK134605 and GINOP-2.3.3-15-2016-00020.

Conflicts of Interest

The authors declare no conflict of interest.

P196

A network-based approach for the analysis and biological interpretation of proteomics data

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Proteomics generates vast amounts of data that need to be properly analyzed and interpreted in biological context. In particular, the data analysis should fully explore the functional information of the proteomics landscape. We have developed a network-based approach to infer proteomics data in the context of molecular interaction networks. Components of this approach are i) a resource, ConsensusPathDB [1], covering ~600,000 protein-protein interactions integrated from 18 different resources, ii) a computational method, NetCore [2], for network propagation to identify functional modules from large omics data. We show how this approach can be used to infer mechanistic information from MS-based protein identification and quantification experiments as well as phosphoproteomics experiments in the context of drug safety. Furthermore, we show that the network approach is an elegant way of integrating different omics data and, thus, generating congruent interpretation from heterogeneous data [3], an important step in systems medicine approaches.

References

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P197

Retrieving peptides by masses in protein-graphs to allow mutationally and variationally altered peptides to be exported into FASTA

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Objective

Currently, spectra-identification relies on FASTA-files and their protein entries. By utilizing so called search engines, spectra can be annotated with peptide sequences for identification. However, FASTA-files mostly contain the canonical- sometimes isoform-sequences of proteins, which limit search engines to only identify these, failing to identify peptides containing variations and/or mutations. To enable search engines to identify such peptides, we developed ProtGraph to generate compact directed acyclic graphs from proteins, by utilizing SwissProt-EMBL entries from UniProtKB. These protein-graphs encode the canonical (and isoform) sequences as well as feature information like mutagen- or variational information and more. To utilize the encoded feature information for spectra-identification, an export from the graph into FASTA-format is mandatory. The huge number of peptides contained in such graphs can be challenging, creating large FASTA-files, so that a more sophisticated approach than a naïve export is needed.

Methods

To tackle this problem, we limit the export of peptides to only peptides, fitting the spectrum masses. In order to retrieve peptides by mass from protein-graphs we introduced a new attribute to the graph-nodes, so called pattern databases (PDBs). These entries, containing multiple intervals of achievable delta-weights, can then be further utilized by partial paths inside the graph to quickly and early filter out paths which are unachievable by the queried mass. Since protein-graphs are directed and acyclic, a topological order can be used for traversing. In combination with PDBs, valid peptide paths can be extracted efficiently. The algorithms performance as well as memory consumption was further optimized by utilizing a specifically ordered topological order.

Results

We illustrate that annotating PDBs can be performed efficiently in quadratic time, showing that such an annotation is feasible for all proteins in UniProtKB. Furthermore, we show that with an arbitrary topological order, mass queries for most proteins containing available feature information can be executed efficiently. By introducing a specific topological order, we show that querying peptides can be further optimized.

Conclusion

Summarized, we provide a solution to query peptides by mass, e.g. mass spectrometrical parent masses, from protein-graphs. By utilizing PDBs with a specific topological order the number of peptide candidates during the traversal for proteins-graphs is reduced immensely. The efficient traversal allows to query almost all protein-graphs from UniProtKB. Only a few exceptions, such as P04637 (TP53), containing up to ~10^220 theoretical peptides, are partially queryable with this approach. Overall, the efficient generation of a FASTA-file from protein-graphs of complete UniProtKB which fit to provided spectra is possible for most proteins, allowing search engines to search for variationally and mutationally altered peptides.

P198

Quantitation capabilities of the ultrafast LC-MS1-only proteomic method DirectMS1: comparison with state-of-the-art DDA and DIA approaches

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Objective

Recently, we presented a DirectMS1 method of ultrafast proteome-wide analysis based on minute-long LC gradients and MS1-only mass spectra acquisition. Currently, the method provides the depth of human cell proteome coverage of 2000 proteins when using 5-min LC gradients. Note that the widely used MS/MS-based approaches provide 4000 to 5000 protein identifications within a couple of hours of instrumentation time. However, on the contrary with the common perception we advocate here that the higher number of identified proteins does not always translate into better quantitation quality of the analysis. To further elaborate on this issue we focused this study on the comparison of DirectMS1 quantitation performance with three current methods

of choice in proteomics: LFQ DDA, TMT-based DDA, and DIA. For the comparison we performed quantitative proteome-wide analysis of well-characterized (ground truth) and real world biological samples.

Methods

The first data set was acquired for a mix of UPS1 proteins spiked at 0.1 to 50 fmol into E. Coli as a background using Orbitrap Fusion Lumos operated in MS1-only mode and 5 min LC gradients. The results of this analysis were compared with the ones published recently for the same UPS+E. Coli samples analysed using 90 min DIA. Next, we analyzed a set of glioblastoma cell lines exhibiting different sensitivity to vesicular stomatitis virus after interferon treatment using 5 min DirectMS1, 120 min MS/MS-based LFQ DDA, 40 min single-shot and 60 min X 10 fractions MS/MS TMT-based DDA methods. DirectMS1 data was analyzed using DirectMS1Quant, a novel quantitation workflow designed specially for DirectMS1 analysis.

Results

TMT analysis of glioblastoma samples using extensive fractionation provided the best results for the glioblastoma data in terms of the number and comprehensiveness of the revealed interferon regulated pathways. The results obtained using DirectMS1 were comparable with TMT, yet, with 10 fold smaller overall instrumentation time spent. Comparison of DirectMS1 and DIA quantitation results for UPS/E.Coli mixture revealed better DirectMS1 sensitivity for lower concentrations of spiked proteins, while DIA quantified more UPS proteins at higher concentrations.

Conclusion

The results obtained have shown that the ultrafast method DirectMS1 of proteome analysis provides comparable quantitation performance proteome-wide with the current state-of-the-art MS/MS-based approaches used in proteomics such as TMT-based multiplexed DDA and DIA. However, DirectMS1 reduces the instrumentation time to perform the proteome-wide analyses 10-fold. It also alleviates the experimental complexity as being a label- and MS/MS-free method.

Financial support from the Russian Science Foundation (grant no. 21-74-10128) and PRO-MS: Danish National Mass Spectrometry Platform for Functional Proteomics (grant no. 5072-00007B)

P199

Democratizing Data-Independent Acquisition Proteomics Analysis on Public Cloud Infrastructures Via The Galaxy Framework

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Data-independent acquisition (DIA) has become an important approach in global, mass spectrometric proteomic studies because it provides in-depth insights into the molecular variety of biological systems. However, DIA data analysis remains challenging due to the high complexity and large data and sample size, which require specialized software and vast computing infrastructures. Most available open-source DIA software necessitate basic programming skills and cover only a fraction of the analysis steps, often yielding a complex of multiple software tools, severely limiting usability and reproducibility. To overcome this hurdle, we have integrated a suite of DIA tools in the Galaxy framework for reproducible and version-controlled data processing. The DIA suite includes OpenSwath, PyProphet, diapysef, and swath2stats. We have compiled functional Galaxy pipelines for DIA processing, which provide a web-based graphical user interface to these pre-installed and pre-configured

tools for their usage on freely accessible, powerful computational resources of the Galaxy framework. This approach also enables seamless sharing workflows with full configuration in addition to sharing raw data and results. We demonstrate the usability of an all-in-one DIA pipeline in Galaxy by the analysis of a spike-in case study dataset. Additionally, extensive training material is provided, to further increase access for the proteomics community. This work has been submitted to GigaScience.

P200

Mistle: Metaproteomic index and spectral library search engine

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Objective: With the introduction of accurate deep learning predictors, spectral matching applications might experience a renaissance in tandem mass spectrometry (MS/MS) driven proteomics. Deep learning models, such as Prosit, predict complete peptide fragmentation patterns including observable peak intensities. This gives the unprecedented opportunity to accurately predict mass spectra that may arise from any given proteome. However, the magnitude of spectral data is huge when querying large search spaces, e.g. derived from many species. For example, the human gut metaproteome, registered in the Integrated Gene Catalog (IGC), covers >350 million peptides, which amounts to over 500 GB of predicted MS/MS data. This makes it unfeasible for current spectral matching software to search such libraries, displaying a need for optimized algorithmic solutions.

Methods: Using a data structure known as fragment-ion index, first introduced by MSFragger, we facilitate an efficient peak matching algorithm for computing spectral similarity between query and library spectra. Mistle (**M**etaproteomic **i**ndex and **s**pec**t**ral **l**ibrary search **e**ngine) uses index partitioning and SIMD intrinsics (Single instruction, multiple data), which greatly improves speed and memory efficency for searching large spectral libraries. This way, Mistle can process terabytes of tandem MS/MS data by building a comparably small index, which allows for an efficient search within a few hours. Mistle is written in C++20 and is highly parallelized.

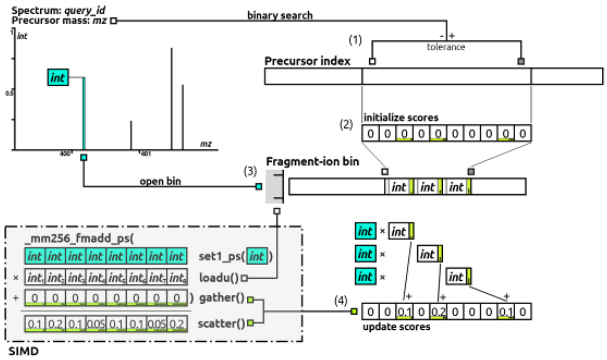
Results: We demonstrate the efficiency of Mistle on fully predicted gut metaproteome catalogued in the IGC. Compared to the commonly used spectral library search engine SpectraST, we show a 75-fold runtime improvement in constructing a searchable index and more than four times speed-up of the spectral search. For large queries, Mistle is faster than the database search engines X!Tandem and ProteoStorm. Furthermore, we find evidence that suggests the matching approach to predicted spectral libraries identifies peptides with higher precision. Mistle detects peptides not found by database search via X!Tandem and in turn reveals priorly unnoticed false discoveries among their matches. We given an outlook on further application fields with large search spaces, such as proteogenomics.

Conclusion: In this study, we show that fully predicted spectral libraries can improve peptide identification. Mistle provides effective means to search large-scale spectral libraries, which we demonstrate for the human gut metaproteome.

Fig. 1: Mistle"s search algorithm. (1) Binary search in precursor index. (2) Initialization of empty scoring vector. (3) binary fragment search in fragment-ion index. (4) Update of scores using SIMD intrinsics.

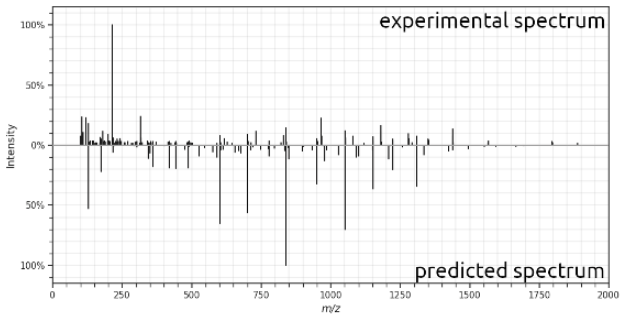
Fig. 2: Example match to the same query spectrum. X!Tandem (upper plot) seemingly finding a mismatch (according to Prosit's prediction) and Mistle (lower plot) identifying a much more plausible peptide.

Fig. 1

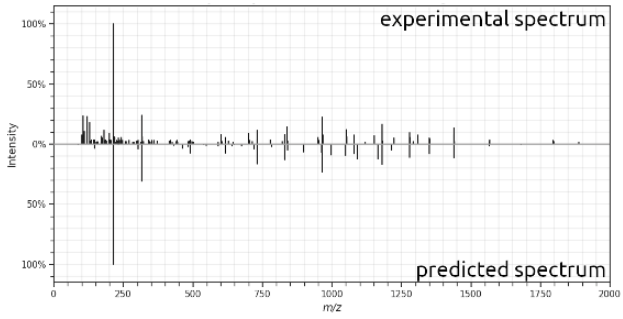


Poster presentation

Fig. 2



Example spectrum matched by **X!Tandem**
(peptide: KSQVFSTAEDNQSAVTIHVLQGER)



Example spectrum matched by **Mistle**
(peptide: SQVFSTAEDNQSAVTIHVLQGERK)

P201

MS²Rescore: Data-driven rescoring dramatically boosts immunopeptide identification rates

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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 and deep 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 combining the new MS²PIP model with DeepLC and Percolator into MS²Rescore we were able to identify 46% more spectra and 36% more unique immunopeptides compared to conventional Percolator rescoring. Furthermore, rescoring with peak intensity and retention time 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 MS²Rescore, updated with a new non-tryptic MS²PIP spectrum prediction model greatly enhances immunopeptide PSM rescoring, resulting in vastly improved identification rates, and allows for 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.

P202

Confirming non-synonymous genomic variation events through multiple coverage at the proteome level

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Question

Can shotgun proteomics provide improved reliability in detection of genomic or transcriptomic events through multiple peptide coverage, akin to Next generation sequencing?

Methods

Distinct peptides which confirm the same single amino acid variants may originate from miscleavages or parallel use of different proteases or cleaving agents. We analyze publicly available multiprotease datasets, as well as own data on HEK-293 proteome using trypsin, GluC and LysC.

In addition to single amino acid changes, we consider peptides that span alternative splice junctions. To this end, we employ the RefSeq Human Genome annotation (GRCh38). We also explore the possibility of "de-novo" identification of previously un-annotated splice variants based on the existing markup of exons in the genome. A combinatorial database of novel junction-spanning peptide sequences is constructed and applied for analysis of melanoma cell line proteomes.

Results

While most of the visible proteome is only covered 1-fold by unique peptides, some discoveries are indeed confirmed by multiple coverage. We consider the relationship between the degree of multiple coverage, the depth of proteome analysis, and the number of proteases using several datasets: deep human brain proteome using trypsin-only and trypsin-LysC digestion, Confetti multiprotease proteome map, and our own HEK-293 proteome with trypsin, GluC and LysC. With the proteome depth varying from five to eight thousand protein groups and the amount of proteases between one and seven, the proportion of multiple coverage within the visible proteome varies from 7% (trypsin only) to 35% (seven proteases). Out of 36 detected genomic variants in HEK-293 proteome, seven were confirmed by multiple coverage. We also found peptide evidence for 4209 alternative splicing events, and were able to calculate the peptide coverage for 3350 of them. 478 alternative splicing events were confirmed by multiple peptide coverage, a lower percentage than the average over the whole proteome.

Conclusion

The use of multiple proteases allows achievement of a high degree of reliability in detection of some genomic events. To increase the degree of multiple coverage, both the number of proteases and the depth of proteome analysis need to be maximized.

Proteogenomic discoveries confirmed by multiple coverage may facilitate the identification of cancer missense mutations which may serve as neoantigens, including experimental schemes of neoantigen vaccine production. Actionable or diagnostic cancer mutations and splice junctions may be identified more reliably with the proposed method, which would make it possible to omit a further validation stage, such as targeted proteomics or antibody-based methods.

The work was supported by the Russian Science Foundation (grant number 20-15-00072) and Russian Basic Science Foundation (grant number 21-34-70020).

P203

Minimizing Identification and Quantification Errors in Proteomics by DIA

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Objective

Data-independent acquisition (DIA) is becoming increasingly popular in quantitative proteomics. However, key data processing strategies are insufficiently characterized by their impact on quantitative accuracy. We use the LFQbench principle[1,2] to benchmark the accuracy of fold-change quantification depending on gradient length, spectral library selection, DIA-NN[3] settings, and post-processing variables.

Poster presentation

Methods

The LFQbench samples A and B consisted of human, yeast, and E.coli digests, with the expected log2 fold changes of 0, +1, and -2, respectively. Per sample type and gradient (30, 90, 150 min), 0.9 µg sample were analyzed 4 times on a Q Exactive HF. Raw data were analyzed with DIA-NN 1.8, and results were processed with an in-house R-script including differential expression analysis by limma[4] ($\alpha < 0.01$). Statistical indicators incl. FDR were estimated using the "confusion matrix" principle of matching limma results against the expected outcome.

Results

The R-script developed in-house is a useful tool to summarize, visualize, and compare LFQbench datasets. It quantitatively describes errors related to normalization, skewness, dispersion, and erroneous peak-to-protein group assignments. We elucidated the impact of various analysis settings or processing variables by comparing the statistical indicators.

Choosing a combination of favorable over default settings (figure 1) results in 38 % more true-positives, a 3 times lower FDR, and negligible fold-change ratio expansion. Each of the 90 min LC-MS/MS gradients lead to detection of ca. 80 000 precursors from over 9000 protein groups. Additionally, the quantitative accuracy compares favorably to LFQbench tests including QE HF-X, diaPASEF, and Scanning SWATH data.[5]

Conclusion

We provide an experiment optimization scheme for highly accurate and reproducible label-free protein quantification by DIA.

References

1 Kuharev et al., *Proteomics* 2015

2 Navarro et al., *Nature biotechnology* 2016

3 Demichev et al., *Nat. Methods* 2020

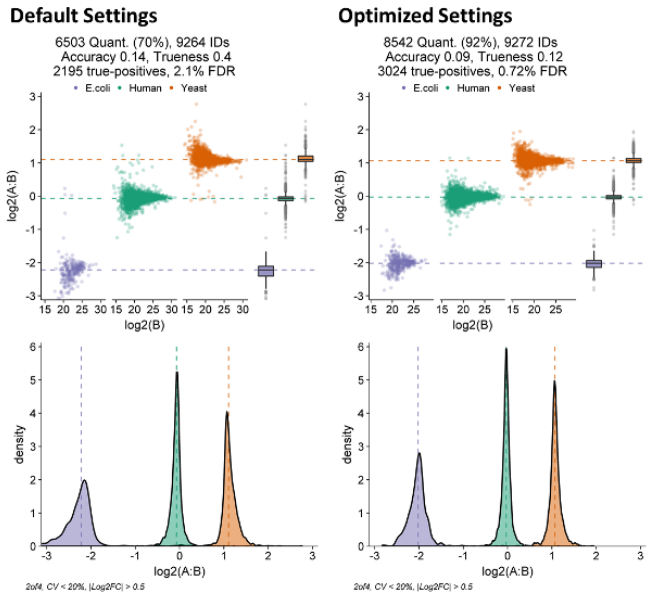
4 Ritchie et al., *Nucleic Acids Res.* 2015

5 Van Puyvelde et al. *bioRxiv* 2021 (PXD028735)

Figure 1: Protein group (PG) level visualization of a 90 min gradient LFQbench dataset analyzed with default and optimized DIA-NN settings.

While setting optimization has little impact on the number of identified protein groups (IDs), it strongly impacts the quantification. Accuracy is the average distance between measured and expected log2 fold-changes, trueness is the cumulative distance between medians (dashed lines) and expected values. Average errors between fold-changes and expectation values are reduced, as well as the rate of aberrant data points, e.g. human PGs with a log2 fold change around +1. Furthermore, yeast and E.coli PGs appear more symmetrical around the expectation values due to the reduced skewness.

Fig. 1



Poster presentation

P204

KNIME workflow for the processing of DIA-NN data

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Objective:

DIA-NN (Demichev et al., 2020) is a widely used tool for analyzing data-independent acquisition (DIA) mass spectrometric (MS) data. However, further processing of outputs from DIA-NN is necessary for acquiring biologically or clinically relevant information. This can be done in the KNIME Analytics Platform (Fillbrunn et al., 2017), an open-source tool which allows building unique workflows using a series of built-in or custom nodes.

Our main objective was to develop a streamlined workflow for further processing of DIA-NN outputs using KNIME.

Materials & Methods:

MS data from a general DIA-MS experiment were first analyzed using the DIA-NN software (version 1.8) and the resulting .tsv file was then used for further processing in KNIME. KNIME was run inside a docker container (https://github.com/OmicsWorkflows/KNIME_docker_vnc; version 4.1.3a). The KNIME workflow was built using built-in nodes as well as custom nodes (available at https://github.com/OmicsWorkflows/KNIME_metanodes). R and Python scripts were used for selected data-processing, visualization and statistics steps.

Results:

The presented workflow is used for processing DIA-NN main output table.

During the initial processing, low quality data are filtered out and grouping of precursors on peptide and protein level is performed. Protein information are extracted from the UniProtKB database and appended to the table. Contaminants are then filtered out and log2 transformation of protein intensities is performed.

There are several options for linear and non-linear data normalization with accompanied quality control (QC) figures, e.g. violin plots, cluster analyses or MA plots. QC figures can be made for data already normalized by DIA-NN. Imputation of missing values can be also performed. Different samples can be compared using LIMMA test, volcano plot is used for data visualization. It is also possible to check and select for processing protein groups defined only by proteotypic peptides or those based on non-proteotypic ones.

Conclusion:

The developed workflow can be used for the processing of DIA-NN outputs using a series of data manipulation, biostatistics and bioinformatics steps. It helps to improve efficiency as well as reproducibility of the process. The workflow "DIA-NN_PGs_LFQ workflow" can be found at https://github.com/OmicsWorkflows/KNIME_workflows.

Acknowledgement:

This work was supported from European Regional Development Fund-Project "SINGING PLANT" (No. CZ.02.1.01/0.0/0.0/16_026/0008446).

Literature:

Demichev, V., Messner, C.B., Vernardis, S.I. et al. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. Nat Methods 17, 41–44 (2020). DOI: 10.1038/s41592-019-0638-x

Fillbrunn A, Dietz C, Pfeuffer J, Rahn R, Landrum GA, Berthold MR. KNIME for reproducible cross-domain analysis of life science data. J Biotechnol. 2017 Nov 10;261:149-156. DOI: 10.1016/j.jbiotec.2017.07.028.

Poster presentation

P205

The landscape of proteomics tools and workflows

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Objective

There has been a myriad of efforts to improve the analysis and interpretation of proteomics data. Given the large number of methods and their high potential, we investigated the state of available and used software in proteomics, and assessed how this knowledge can be used to build new data analysis workflows. For better testing and more immediate usage of computational workflows, we aim to build a widely applicable cloud-based framework for analysis, tool testing and benchmarking.

Methods

We statistically assessed the software registry bio.tools for the number of available tools in proteomics. Annotations about tool functionality in bio.tools were used for the automatic composition of proteomics data analysis workflows by the Automated Pipeline Explorer.

Five different workflows were implemented in the NextFlow framework to analyze label-free MS data in a reproducible and scalable manner, available through the WOMBAT-P repository at https://github.com/wombat-p.

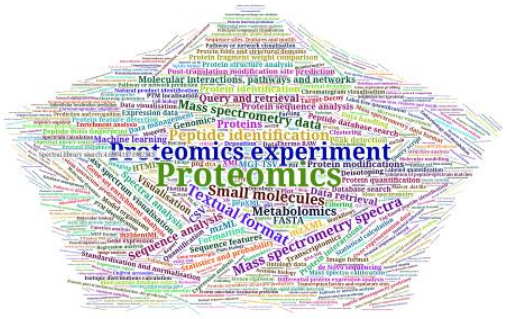
Results

There are currently more than 1,500 proteomics-associated tools registered on bio.tools, including a wide variety of functions and intersections with other fields. Automatic workflow composition from this wealth of information shows promising results but still suffers from missing details and lack of sufficient interoperability between different tools. By relying on established tool combinations, five WOMBAT-P workflows are now ready for testing and benchmarking, and will be prepared to re-analyze large amounts of public data from the PRIDE data repository by processing the new metadata format SDRF.

Conclusions

We obtained a detailed view on the tool landscape in proteomics. Given the large number of tools, integration and testing of new algorithms by incorporation in established or alternative workflows is still a major stumbleblock. Our workflows and the associated environment will relieve this burden. With a soon available interface to run them on the cloud, our open-source approaches will provide more appropriate solutions for a specific data analysis in a faster pace. Their applicability will be shown on a large body of public data as part of an implementation study of the ELIXIR infrastructure consortium.

Fig. 2



P207

Proteograph Analysis Suite: A cloud-scalable software suite for proteogenomics data analysis and visualization

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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.

P208

Identification of Protein-Protein Interactions on Mitochondria Outer Membrane by Crosslinking Mass Spectrometry

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Almost all mitochondrial proteins are nuclear encoded, translated in the cytosol and guided to the mitochondrial outer membrane, i.e. surface receptors, which direct their cargo proteins through sophisticated protein import machineries into the outer membrane or to appropriate subcompartments of mitochondria. In this process, various protein complexes play an important role through different modes of action in order to obtain diverse topologies as well as localisations, such as membrane associated, peripheral or transmembrane proteins. During this process, proper conformation of cargo proteins is facilitated by specific cytosolic import factors. Therefore, protein-protein interactions (PPIs) have an essential role in these processes, and are subject of various studies. Crosslinking mass spectrometry (XL-MS) is a promising method, which plays a growing role for structural (structural constraints and topology) and pathway studies (interactomics) based on the PPIs. Principally, XL-MS is performed through three steps, in which the interacting proteins are first isolated, then crosslinked as well as separated and finally measured by MS. However, in XL-MS analysis of membrane proteins, the isolation of crosslinked membrane proteins is challenging due to the insolubility of membrane proteins under native conditions. Here, we first crosslinked the membrane proteins using BS3 on isolated intact mitochondria, and fractionated them using Triton-X114. After LC-MS/MS analysis, we compared these results with the data originating from *in-gel-crosslinking* using BN-PAGE. Our data suggest reproducible and comparable results regarding the PPIs of mitochondrial membrane proteins and reveals unknown interaction partners of novel mitochondrial MPs, such as SMIM26.

P209

Proteomics study of lymphocytes cargo as liquid biopsy in COVID-19 patients

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Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) is a highly transmissible and pathogenic beta-coronavirus that caused a pneumonia pandemic named Covid-19 from late 2019, generating a profound health, economic and social crisis around the world. The surprising variability of clinical manifestations, based on the marked reduction in lymphocyte

subgroup counts, cytokine storm, axonal damage and hypercoagulopathy, highlights the urgency to characterize the molecular networks involved in Covid-19. In this context, peripheral lymphocytes bring the cellular basis of adaptative immune responses, thus playing a considerable role as predictors of Covid-19 outcomes. In this work we have synergistically combined the shotgun proteomics approach on CD3+ T lymphocytes and CD19+ B lymphocytes isolated and purified by FACS (reaching a purity> 95%) starting from the plasma of hospitalized patients during infection (**I**), recovered (**R**) and healthy subjects (**H**). Proteins were digested by filter-aided sample preparation (FASP) and peptides were separated on an EASY Spray C18 analytical column before nano-LC-Orbitrap-Fusion-Tribrid-MS analysis, obtaining 221 (**I**), 165 (**R**) and 234 (**H**) proteins in isolated CD3+ T cells, and 205 (**I**), 118 (**R**) and 161 (**H**) proteins in CD19+ B cells. Among the most interesting differences, we found that samples (**I**) lacked effective lung healing markers as CD44, but expressed markers of cytoskeleton remodeling (MMP9), inflammation (ORM2), proteasomal activity (PSMB6), virus entry (HSPA9 and glycophorins), and coagulation (PF4). Furthermore, we found upregulated levels of transferrin (TF), known as a procoagulant, in COVID-19 patients, demonstrating how the disease is affected by iron homeostasis. Moreover, PF4 expression was individually validated on all patients through cytofluorimetry analyzes. Furthermore, IPA Core Analysis highlighted some upstream regulators related to coagulation, inflammation, oxidative stress and neuronal homeostasis, suggesting a close interaction between inflammation, viral infection and coagulation cascade and demonstrating how this information is conveyed by the protein cargo of B and T lymphocytes circulating in patients with SARS-Cov-2. As a sort of liquid biopsy, our pioneering application of cell SORTomics could provide useful tools in assessing the response to Covid-19 infection and in predicting its outcomes.

P210

Inferring context-specific signalling states using large-scale CRISPR-KO screens

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Cellular signalling drives in large parts the interaction of a specific cell with its environment. The context of a cell, which can be anything that characterises that cell like tissue of origin or the mutational landscape, strongly influences the specific wiring of the signalling network. Studying these context-dependent signalling states is crucial to understanding the phenotype and behaviour of cancer cells in different contexts. This knowledge can be leveraged to propose new drug therapies in a personalised manner.

Currently protein-protein interaction networks best capture our understanding of signalling networks, hence we sought to use interactomes and integrate them with genome-wide CRISPR knockout data from various cancer cell lines to derive context-specific signalling networks.

Large-scale CRISPR knockout screens covering a wide array of cell lines, for example DepMap and Project Score, revealed that many genes are essential in only a subset of cell lines. The challenge is to systematically identify and define those contexts and how they affect gene interactions and cell signalling. Using CEN-tools (https://doi.org/10.15252/msb.20209698), we tried to identify context-specific parts of the cell signalling network. To this end, we built a linear model to disentangle the tissue-specific effects of different driver gene mutations on gene essentiality. Contrary to expectations, we observe that the genes essential in the context of a cancer driver mutation are highly tissue-specific. By mapping those genes onto a protein-protein interaction network we were able to extract context-specific essential signalling modules. These findings could lend insights into why drugs targeting the same cancer driver are effective in cancer of one tissue type but not in another. Furthermore, they can help identifying synthetic lethalities to aid drug development for targeted therapy.

Poster presentation

P211

Mitochondrial protein biogenesis in the synapse is supported by local translation

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[6]*International Institute of Molecular and Cell Biology, Laboratory of RNA Biology, Warsaw, Poland*

Synapses are the regions of the neuron that enable the transmission and propagation of action potentials at the cost of high energy consumption and elevated demand for mitochondrial ATP production. The rapid changes in local energetic requirements at dendritic spines imply the role of mitochondria in the maintenance of their homeostasis. Using TMT and LFQ approach proteomic analysis supported with complementary experimental approaches, we show that an essential pool of mitochondrial proteins is locally produced at the synapse indicating that mitochondrial protein biogenesis takes place locally to maintain functional mitochondria in axons and dendrites. Furthermore, we show that stimulation of synaptoneurosomes induces the local synthesis of mitochondrial proteins that are transported to the mitochondria and incorporated into the protein supercomplexes of the respiratory chain. Importantly, in a mouse model of fragile X syndrome, *Fmr1* KO mice, a common disease associated with dysregulation of synaptic protein synthesis, we observed altered morphology and respiration rates of synaptic mitochondria. That indicates that the local production of mitochondrial proteins plays an essential role in synaptic functions. We also used surface labeling of synaptoneurosomes, to confirm it receptors are recruited to the synaptic membrane

P212

Semi-automated and high-throughput homogenization technique for in-depth analysis of various tissue proteome

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Introduction:

Efficient protein extraction is a crucial and challenging step in tissue sample preparation for LC-MS analysis. While conventional methods are faced with various challenges - such as low-throughput and time-consuming workflows - our novel tissue lysis workflow on the semi-automated "BeatBox" platform enables efficient protein extraction for 96 samples in parallel in as little as 10 minutes. Coupled to the robust iST technology that is fully automatable on common liquid handling platforms, we combined in-depth sample preparation for large-scale applications with minimal hands-on time.

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 bead-based technologies, a defined energy input is applied to each sample facilitating highly efficient protein extraction. For complete nanoLC-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

BeatBox platform speed-up and simplified the tissue preparation for proteomics experiments. From intact tissue sample to data visualization it takes less than 4 hours. We identified ~2300 proteins from mouse heart muscle, ~3000 protein from mouse liver tissue and >4000 protein from mouse lung tissue. In comparison to traditional iST workflow, we gain up to 13% more protein identifications. At the same time, excellent technical variability (e.g. for mouse liver: median R^2 of 0.98 and median CV of 9.2 %) was obtained. Furthermore, a combination of the ´BeatBox" iST workflow with tip-based peptide fractionation increased the protein identifications by 40 % compared to unfractionated samples.

Poster presentation

Conclusion:

The innovative "BeatBox"-based workflow will set a new standard in large-scale tissue sample preparation by enabling ultra-fast and highly efficient protein extraction in a high-throughput manner.