



MEDICAL UNIVERSITY
OF VIENNA

Austrian Proteomics and Metabolomics Research Symposium (APMRS) & Central and Eastern Proteomic Conference (CEEPC)

Building bridges in Science –

Metabolomics and Proteomics between East and West



Book of Abstracts

September 23th - 25th 2024

Medical University of Vienna

Van Swieten Hall of the MedUni Vienna

Van-Swieten-Gasse 1a
1090 Vienna



Welcome to the APMRS & CCPC 2024!

Dear Participants,

It is our great pleasure to welcome you to this year's APMRS, which is being held for the first time together with the CEEPC. This event is organized to foster collaboration, knowledge exchange, and innovation across borders, "Building Bridges in Science—Metabolomics and Proteomics between East and West."

In today's rapidly evolving scientific landscape, it is more important than ever to break down barriers and connect ideas from different regions and perspectives. Our goal is to bridge the scientific communities of Eastern and Western Europe, advancing metabolomics and proteomics research through shared expertise, technology, and insights.

We hope this conference inspires new partnerships and discoveries that will shape the future of proteomics and metabolomics research in Austria and Europe.

Thank you for joining us, and we look forward to an enriching and fruitful exchange.

Warm regards,

Klaus Kratochwill and the organizing team

Organizing Team

Klaus Kratochwill
Medical University of Vienna

Evelyn Rampler
University of Vienna
APMA President

Marcel Kwiatkowski
University of Innsbruck
APMA Vice President

Tamara Tomin
TU Vienna
APMA Secretary

Martina Marchetti-Deschmann
TU Vienna

Rebecca Herzog
Medical University of Vienna

Suresh Jivan Gadher
CEEPC



WELCOME ADDRESS FROM CEEPC 2024

Dear Delegates

On behalf of the CEEPC, we have great pleasure in welcoming you all to the 18th Central and Eastern European Proteomic Conference organized jointly together with APMA, the Medical University of Vienna (MedUni Wien) and TU Wien.

We are confident that this conference will once again immerse everyone in excellent ‘Systems Biology’ incorporating proteomics, metabolomics, and multi-omics. We are privileged to have the presence of eminent speakers from different countries, who will share their expertise each day. In keeping with the CEEPC ideology, we warmly welcome young researchers who will share their novel ideas and fascinating research for all to benefit from.

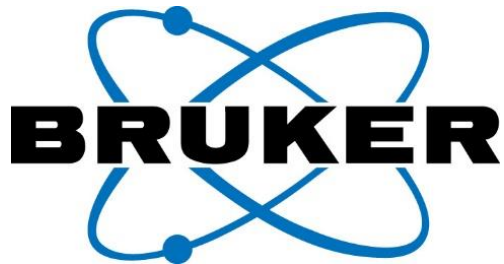
A multidisciplinary program encompassing ‘cutting-edge’ science, enabling technologies, novel software, AI / Machine Learning and the role of Mass Spectrometry in advancing our understanding of protein functionality in biomedicine is there for all to enjoy. We welcome all participants to share their excitement and urgencies of many diverse scientific, clinical and proteomic challenges of the day, the central aim being translation of potential findings into viable solutions and possible therapies for diseases affecting mankind.

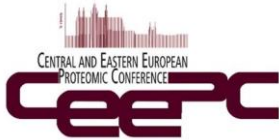
We are sure that the academic heritage of Vienna, will infuse a sense of pursuit of excellence over the next few days and that the city's grand architecture, palaces, and cultural heritage, will provide a magical charm and rich memories to take back home. Furthermore, CEEPC hopes you will enjoy and imbibe the Viennese hospitality together with a specially tailored social programme, whilst actively engaging in productive interactions, networking and friendship.

We wish you a very fruitful conference and a pleasant stay

Suresh Jivan Gadher - on behalf of CEEPC

Thanks to all our Sponsors!





The Central and Eastern European Proteomic Conference (CEEPC)

The Central and Eastern European Proteomic Conference has progressed to its 18th Conference in Central and Eastern Europe with links to international institutions worldwide to facilitate expert scientific interaction and collaborations. An informative website at <http://ceepc.eu/> not only distinguishes CEEPC from other proteomic organizations but also underlines the uniqueness and individuality of its ethos and ideology. The birthplace of CEEPC together with advancing proteomics is captured by the CEEPC *logo showing the ascending spires of the city of Prague* outlined by the intensity of protein / peptide peaks of mass spectrometry depicting the pinnacles of excellence and cohesion of the CEEPC community.

The initial vision of a forum for enthusiastic scientists and researchers to meet and discuss their work in a relaxed manner in middle sized meetings remains unchanged to this day. Rotation of the meeting's venue each year to cultural cities of the world such as Prague, Vienna, Budapest, Jena, Poznan, Kosice, Bucharest, and Vilnius, adds to the intertwining of 'cutting edge' research and the excitement that goes with it.

CEEPC's success is not only due to it embracing emerging topics in proteomics such as 'single cell proteomics' but also due to encompassing of 'multi-omics', 'spatial – omics' and appreciation of hot research topics for advancing science and medicine.

Proteomic technologies have progressed over the last decade allowing in principle, the comprehensive analysis of expressed proteins in time and space. Until now, quantitative proteomics has been pin-pointing minor differences in the protein levels between normal and pathological samples. There is now an urgent need for sophisticated '*enabling technologies*' to identify structural differences in proteins introduced by mutations or structural variations induced by post-translational modifications or protein truncation that are associated with a disease. Additionally, comprehensive characterization of the small molecule metabolites in the biological systems and biological applications of the *Metabolome* together with the *Proteome* in *Precision Medicine* of the patient, stands to revolutionize global health.

The complexity of the data generated has also been a stumbling block in understanding diseases because proteome analysis does not provide a simple 'yes/no' answer but rather requires deep interpretation. To this end, utilization of data from various 'multi-omics' studies including proteomics, genomics, and metabolomics, together with AI / Machine Learning in the hands of skilled researchers, can have significant impact in understanding the disease state.

CEEPC's careful balance between excellence and focused *societal needs*, also holds the key to its success. CEEPC raises concerns about humanitarian issues whilst addressing societal needs, may it be mental health affecting today's generation or the need for an effective vaccine for Ebola, MPox, or Malaria. CEEPC does not shy away from highlighting humanitarian issues such as malnutrition in poor countries, a need for clean drinking water, sanitation and basic education for its young generations.

Suresh Jivan Gadher

Acknowledgement:

We are indebted to the CEEPC Community, the CEEPC Scientific committee and the Austrian Proteomics and Metabolomics Association (APMA), the Medical University of Vienna (MedUni Wien) and TU Wien – our sincere Thanks to all.

Monday 23. September 2024

Session+Chair	Start			
	13:00	Welcome	Klaus Kratochwill, Evelyn Rampler (APMRS) & Suresh Gadher (CEEPC)	
S1 - Ruth Bimer-Grünberger, Isabella Burger	13:15	Invited Lecture	David Gomez (AT)	Deciphering the Microbiome/Host Interactions using Mass Spectrometry-based Proteomics
	14:00	Oral Presentation 1	Fabian Eibensteiner (MedUni Vienna)	Solid organ metapeptidome reveals genuine detection of microbial translocation in a murine leaky gut model in comparison to 16S rRNA sequencing
	14:20	Oral Presentation 2	Rupert Mayer (IMP Vienna)	Reanalysis of immunopeptidomics data reveals novel bacterial epitopes.
	14:40	Oral Presentation 3	Anna-Lena Mayr (VetMed Vienna)	Factors of metronidazole resistance in <i>Trichomonas vaginalis</i>
	15:00	Industry Talk	Maurine Fucito (Evosep)	Standardized, fully automated plasma workflows enabled by the Evotip Pure
	15:15	Coffee and Posters		
S2 - Evelyn Rampler, Anna-Sophia Egger	16:00	Oral Presentation 4	Fränze Müller (IMP Vienna)	Breaking Barriers in Crosslinking Mass Spectrometry: Enhanced Throughput and Sensitivity with the Orbitrap Astral Mass Analyzer
	16:20	Oral Presentation 5	Juan Manuel Sacnun (MedUni Vienna)	Investigating Dynamic Cellular Crosstalk and Pathomechanisms in Experimental Peritoneal Dialysis through secretomics analysis
	16:40	Oral Presentation 6	Lukas Schmidt (MedUni Vienna)	Effects of Alirocumab on Lipids and ApoB-Containing Lipoproteins Beyond LDL-C
	17:00	Oral Presentation 7	Isabella Burger (TU Vienna)	Discovery of a novel antifungal compound, ilicicolin K, through genetic activation of the ilicicolin biosynthetic pathway in <i>Trichoderma reesei</i>
	17:20	Invited Lecture	Elisabeth Varga (AT)	Metabolite profiles and diversity of different harmful algal bloom forming microalgae
18:05-19:30	Welcome Reception			

Tuesday 24. September 2024

Session+Chair	Start			
S3-Klaus Kratochwill, Tamara Lang	09:00	Invited Lecture	Christoph Messner (CH)	High-Throughput Proteomics for Biomarker Discovery and Precision Medicine
	09:45	Oral Presentation 8	Besnik Muqaku (Ulm, Germany)	Peptidomic analysis of cerebrospinal fluid samples reveals new biomarker candidates for amyotrophic lateral sclerosis
	10:05	Oral Presentation 9	Thomas Mair (Hamburg, Germany)	Thoracic aortic diseases: Identification of diagnostic biomarkers using proteomic analysis
	10:25	Coffee and Posters		
S4 - Marcel Kwiatkowski, Suresh Gadher	11:05	Invited Talk	Boryana Petrova (Harvard, US)	Leveraging Metabolomics to Characterize Embryonic CSF During Inflammation
	11:45	Oral Presentation 10	Christina Brenner (Uni Vienna)	Less is More: Enhancing Glioblastoma Metabolomics by Minimizing Tissue Requirements
	12:05	Industry Workshop	Valeriia Kuzyk (Bruker)	Eliminating the throughput/sensitivity compromise with 4-D proteomics at PASEF speed: current and upcoming applications
	12:50	Lunch Break		
S5-Martina Marchetti- Deschmann, Sophie Honeder	13:50	Invited Lecture	Kristina Swamborn (DE)	MALDI Mass Spectrometry Imaging – Applications in Pathology
	14:35	Oral Presentation 11	Antonia Malissa (TU Vienna)	Parchment – A Collagen Sample: MALDI MS, FTIR and Raman Imaging to Study Localised Proteome and Lipidome Changes
	14:55	Oral Presentation 12	Klára Brožová (MedUni Vienna)	Integrating MALDI-MSI and imaging mass cytometry to reveal intratumoral heterogeneity in breast cancer subtypes induced by the tumor microenvironment
	15:15	Oral Presentation 13	Hartmut Schlüter (Hamburg, Germany)	Proteoforms - how do we get them intact out of tissues?
	15:30	Coffee Break		
S6- Tim Causon, Laszlo Drahos	16:10	Oral Presentation 14	Nikolai Huwa (Dübendorf, Switzerland)	Targeted (Phospho)Proteomics Reveals mTOR Pathway Responses to Growth-Impacting Conditions in Zebrafish PAC2 Cells
	16:30	Oral Presentation 15	Anna Roszkowska (Gdańsk, Poland)	Novel microsampling approach based on SPME probes for monitoring the levels of endocannabinoids and phytocannabinoids in biological samples
	16:50	Industry Talk	Eppendorf	tbd
	17:05	Oral Presentation 16	Amanda Schütz (Uni Vienna)	Development of a new glycolipid spectral library for high-resolution mass spectrometry workflows
	17:25	Invited Lecture	Lilla Turiak (Budapest, Hungary)	Comparing the proteomic and glycomic profiles of A549 and BEAS-2B cell line-derived small extracellular vesicles
	18:10	General Assembly APMA		
	19:00	Conference Party at Venue		

Wednesday 25. September 2024

Chair	Start			
S7 - Micha Birklbauer, Magdalena Luczak	09:00	Invited Lecture	Piotr Widlak (PL)	Proteomics of extracellular vesicles: proteome of melanoma-derived and lymphocyte-derived exosomes from plasma of melanoma patients
	09:45	Oral Presentation 17	Matthias Schittmayer (TU Vienna)	Activity-based tissue atlas of murine serine hydrolases in dependence of nutritional state
	10:05	Oral Presentation 18	Fabian Frommelt (CeMM Vienna)	Mapping of the human solute carrier transporter protein interaction landscape
	10:25	Industry Talk	Covaris	Confident Data with Robust and Reliable Kits and AFA Based Workflows – For Every Proteomics Laboratory
	10:40	Coffee and Posters		
S8 - Karl Mechtler, Marcel Kwiatkowski,	11:20	Oral Presentation 19	Bente Siebels (Hamburg, Germany)	Assay for quantitative and qualitative analysis of adsorption events to uncover loss of molecules during sample handling
	11:40	Oral Presentation 20	Zoltán Szabó (Szeged, Hungary)	Diving deeper in the sea of tears: lessons learnt from the proteomic analysis of a highly variable body fluid
	12:00	Oral Presentation 21	Micha Birklbauer (FH Hagenberg)	Proteome-wide Non-Cleavable Crosslink Identification Using Sparse Matrix Multiplication with MS Annika 3.0
	12:20	Oral Presentation 22	Julia Höhlschen (TU Vienna)	Investigating the cardioprotective effects of SGLT-2 inhibitors
	12:40	Lunch Break		
S9 - Tamara Tomin, Rupert Mayer	13:25	Invited Lecture	Jürgen Hartler (AT)	LipidProphet: a universal confidence metric permits unsupervised large-scale lipidomics
	14:10	Best of APMA	Young Investigator Awards /Society Medals	Short Presentations of the Award Winners
	15:00	Awards and Closing	incl. INFO on next year CEEPC and APMRS	

Invited speakers

Monday 23. September 2024

David Gomez, University of Vienna

David Gómez-Varela is a Scientist at the Division of Pharmacology and Toxicology, University of Vienna. He earned his PhD in 2003 in Spain, studying ion channels using biophysical, molecular biology, and mathematical tools. He completed postdoctoral training in neurobiology at the Max Planck Institute and the University of California San Diego, where he utilized single-particle imaging, electrophysiology, and MS to understand synaptic plasticity. David led the Systems Medicine Innovation laboratory at the Max Planck Institute until 2021, where his team developed technological solutions for individualized molecular fingerprints, including the first PCR-pocket device and a disease-outcome prediction algorithm in COVID-19 pandemic. His current research focuses on the role of the human microbiome in health and disease using mass spectrometry, pioneering the field of metaproteomics in Austria.

Elisabeth Varga, University of Veterinary medicine Vienna

Elisabeth Varga studied "Safety in the Food Chain" at BOKU University where she also obtained her PhD in the area of mycotoxin research. After several research stays abroad and working at the Faculty of Chemistry of the University of Vienna for almost five years, she is currently an assistant professor in the area of Analytical Chemistry with focus on Food and Environmental Analysis at the University of Veterinary Medicine, Vienna. She is working at the interface of analytical chemistry and toxicology with liquid chromatography coupled to mass spectrometry as her primary technique of choice. Beside small molecule biotoxins, her interests are potentially ichthyotoxic microalgae in particular from the genera *Prymnesium*, *Karlodinium* and *Alexandrium*.

Tuesday 24. September 2024 (morning)

Christoph Messner, University of Zürich

Christoph Messner is an Assistant Professor for Precision Proteomics at the University of Zürich and head of the Precision Proteomics Center at the Swiss Institute of Allergy and Asthma Research (SIAF). Christoph completed a PhD in analytical chemistry at the University of Innsbruck in 2015. He undertook postdoctoral training in Markus Ralser's group at the University of Cambridge and at the Francis Crick Institute in London. Christoph then worked for Biognosys as a scientific project manager before joining the University of Zürich in 2022. Christoph's research focuses on the development of high-throughput proteomics technologies and its applications in biomarker discovery and systems biology.

Boryana Petrova, Harvard Medical School/Medical University Vienna

Dr. Petrova is a broadly trained molecular biologist with extensive expertise in LCMS and metabolomics. She worked as a metabolomics expert at prestigious institutions such as MIT and Harvard Medical School (HMS) as well as a lecturer at Northeastern University and the Cambridge Centre for International Research. Recently, Dr Petrova assumed the position of Director of the Research Metabolomics Core Facility at MedUni Vienna. As a postdoc at MIT, research on *Drosophila* oogenesis and *Toxoplasma gondii* drug resistance sparked her interest in metabolomics and LCMS. At Boston Children's Hospital and HMS, as a staff scientist, she established the metabolomics mass spectrometry facility for the lab of Dr. N. Kanarek. She applied metabolomics to various biological inquiries, including cancer, neuroinflammation, developmental biology and more, both in basic and clinically relevant settings.

Tuesday 24. September 2024 (afternoon)

Kristina Schwamborn, Technical University Munich

Kristina Schwamborn is a consultant at the Institute of Pathology, TU Munich, Germany. She received her M.D. from Heinrich-Heine-University in Dusseldorf, Germany. Dr. Schwamborn also holds a PhD from RWTH Aachen University for developing different proteomic assays in search for prostate and bladder cancer biomarkers. Between 2008-2010 she joined the group of Prof. Richard Caprioli in the Department of Biochemistry, Vanderbilt University to utilize imaging mass spectrometry in different clinical applications including diagnosis and risk stratification in prostate cancer. In 2010 she joined the Institute of Pathology, TU Munich and took her board examination in Anatomical Pathology in 2015. Her current research focus centers on the application of MALDI imaging mass spectrometry in pathology diagnosis and risk stratification.

Lilla Turiák, HUN-REN Research Centre for Natural Sciences

Lilla Turiák is a senior research scientist and recently appointed group leader of the Glycan Biomarker Research Group of the HUN-REN Research Centre for Natural Sciences. She obtained her PhD degree from Semmelweis University in Budapest, Hungary working on the proteomics analysis of extracellular vesicles. Between 2013 and 2015 she conducted postdoctoral research at Boston University in Prof. Joseph Zaia's group developing a serial enzymatic digestion method to analyze various compounds from tissue surfaces for subsequent HPLC-MS analysis. Her recent research focuses on the proteomics, glycoproteomics, phosphoproteomics and glycosaminoglycan analysis of small tissue sections and biopsies as well as extracellular vesicles isolated from lung cancer cell lines using mass spectrometry to identify molecular alterations occurring in lung cancer.

Wednesday 25. September 2024

Piotr Widlak, Medical University of Gdańsk

Prof. Piotr Widlak holds an M.Sc. in Molecular Biology, a Ph.D. in Biomedicine, a habilitation in Biochemistry, and a professorship in Medical Sciences. He conducted post-doctoral research at the Karolinska Institute and UT Southwestern Medical Center. In 1997, he joined the Maria Skłodowska-Curie National Research Institute of Oncology, serving as Deputy Director for Research and Chairman of the Center for Translational Research and Molecular Biology of Cancer. Since 2022, he has been the Director of the Clinical Research Support Centre at the Medical University of Gdańsk. Prof. Widlak has authored around 200 peer-reviewed papers, focusing on molecular oncology, particularly cellular stress responses and the role of extracellular vesicles in cancer.

Jürgen Hartler, University of Graz

Jürgen Hartler is a bioinformatician specializing in the development of algorithms and tools for mass spectrometry data analysis, particularly for metabolomics and lipidomics. He has been awarded a DOC and Max Kade fellowship by the Austrian Academy of Sciences, the latter allowing him to work at the LIPID MAPS laboratory at UC San Diego. His achievements in lipidomics were recognized by the Stefan Schuy award (ÖGBMT) and the Mattauch-Herzog award (DGMS). Currently, he is an assistant professor at the University of Graz, heading the Computational Pharmacology group and serving as deputy director of the Doctoral School of Pharmaceutical Sciences. He also focuses on MS data annotation, lipidomics data standardization, and quality assessment methods.

Short Talks

Short Talk 1

Solid organ metapeptidome reveals genuine detection of microbial translocation in a murine leaky gut model in comparison to 16S rRNA sequencing

Fabian Ebensteiner¹, Markus Unterwurzacher², Anja Wagner², Christoph Aufricht¹, Rebecca Herzog¹ and Klaus Kratochwill^{1,2}

1) Division for Pediatric Nephrology and Gastroenterology, Department for Pediatrics and Adolescent Medicine, Medical University of Vienna, Vienna, Austria

2) Core Facility Proteomics, Medical University of Vienna, Vienna, Austria

Introduction

Many extraintestinal noncommunicable diseases (e.g., chronic kidney disease, CKD) interfere with the intestinal immunity. Consequently the immune system is no longer capable to maintain physiological control of the gut microbiome, leading to dysbiosis. CKD causes uremia and systemic inflammation, which are associated with cardiovascular morbidity and mortality. Current pathophysiological understandings of systemic inflammation in CKD involve induction by uremic toxins, oxidative stress, and diminished cytokine excretion/degradation by the failing kidneys. Studies on experimental uremia in rats showed increased bacterial translocation from the intestine to the liver, spleen, and mesenteric lymph nodes alongside higher levels of serum cytokines. The combination of gut dysbiosis and bacterial translocation might play an important role in the pathophysiology of systemic inflammation in CKD. 16S rRNA sequencing, commonly used for microbiome studies, is challenging in low-biomass samples (such as solid organs), as (cross-)contamination of DNA may easily occur. We therefore aimed to analyse the solid organ metapeptidome in a murine leaky gut model and to compare it to the current standard practice of microbiome analysis.

Methods

N=9 mice received 5/6 nephrectomy and were kept alive for 7 weeks to induce CKD. Livers were harvested, snap frozen, and stored in liquid nitrogen in sterile containers. Samples were bead-homogenized (60 mg) and analyzed using 16S rRNA amplicon sequencing, or enzyme digested (Trypsin/LysC), labeled with TMT-18plex, high-pH offline fractionated (36 fractions), and run on a Ultimate 3000 RSLC nano/ Exploris 480 with FAIMSpro, using self-packed reversed-phase C18 column (50 cm/75 µm i.d.). Bacteria FASTA were created from uniprot.org (Reviewed Swiss-Prot, filtered by Taxonomy 2), analyzed in Proteome Discoverer with a consensus run against mouse and bacteria FASTA simulatenously. Only master and master candidate peptides with high confidence were retained, after exclusion of all possible murine peptides. Taxonomy search was done with Unipept (lowest common ancestor) and only taxa with ≥3 distinctive peptides were retained. For sensitivity a BLAST analysis for all possible taxons for each peptide was conducted.

Results and Discussion

A total of 8 vs 19 distinct phyla were detected by our solid organ metapeptidomic approach in a murine leaky gut model. Overall, the solid organ metapeptidome and 16S rRNA sequencing analysis displayed a marked overlap, especially regarding the most abundant phyla, being: Pseudomonadota, Bacillota, Actinomycetota. Metapeptidomic analysis further revealed reduced inter-animal variability of relative abundances for each phylum. BLAST analysis indicated robust assignment of peptide sequences to microbial phyla. The large overlap between different phyla detected from DNA and peptide fragments underlines the synergistic robustness and importance of orthogonal analysis methods to explore genuine microbial signals in low-biomass samples, such as solid organs. It further strengthens the hypotheses and advances the current pathophysiological understanding of systemic inflammation in leaky gut syndrome, as elicited by CKD.

Innovative aspects

- For the first time we describe a robust solid organ metapeptidomic approach in an experimental leaky gut animal model
- Our findings underline the importance of orthogonal multi-omic analysis of low-biomass samples to derive genuine microbial signals

Short Talk 2

Reanalysis of immunopeptidomics data reveals novel bacterial epitopes.

Rupert L. Mayer^{1,2,3}, Karl Mechtler^{1,2,3}

1) Research Institute of Molecular Pathology (IMP), Vienna BioCenter, Vienna, Austria.

2) Gregor Mendel Institute of Molecular Plant Biology (GMI), Austrian Academy of Sciences, Vienna BioCenter (VBC), Vienna, Austria.

3) Institute of Molecular Biotechnology (IMBA), Austrian Academy of Sciences, Vienna BioCenter (VBC), Vienna, Austria.

Introduction

Both viral pathogens such as SARS-CoV-2 and antibiotic-resistant bacterial pathogens such as MRSA (methicillin-resistant *Staphylococcus aureus*) are reemerging as major global health concerns. In order to develop efficient and long-lasting immunotherapies, knowledge about pathogenic antigen and epitope targets is of utmost importance and can be gathered using immunopeptidomics. This technique features immunoprecipitation of peptide-carrying MHC surface molecules from infected cells and LC-MS to identify said epitopes and antigens. However, in contrast to classical trypsin-based proteomics methods, immunopeptides suffer from low abundance as well as reduced isolation and fragmentation efficiency. Conventional search engines such as MaxQuant's Andromeda or Mascot are therefore not well suited to analyse immunopeptidomics data, and even for the gold standard in the field of immunopeptidomics, PEAKS Studio, continuous development is ongoing.

Methods

In this work, a previously published study from Mayer et al. 2022 is reanalyzed with i) PEAKS Online 11, and ii) Proteome Discoverer 3.1, Sequest HT and INFERYS rescoring. The original work used PEAKS Studio 10.5 and focuses on immunopeptidomics analyses of cells infected with the intracellular bacterium *Listeria monocytogenes*. The identified novel bacterial antigens were then further used as vaccine formulations. In line with the initial study, the raw data were searched here against a combined human and *Listeria* database comprising a total of 23,260 entries. Further search details included unspecific digestion and peptide length restriction to 8-30 amino acids with the false discovery rate (FDR) being controlled at 1% on PSM level.

Results and Discussion

Reanalysis of the published data resulted in an increased number of identified immunopeptides particularly for PEAKS Online 11 with a gain of around 30%, while the gain in peptide IDs for INFERYS rescoring was less pronounced. For both, PEAKS Online and INFERYS, the identified immunopeptides displayed the expected peptide length distributions as well as sequence motifs, confirming that the vast majority of the identified peptides are indeed *bona fide* immunopeptides. Both software tools also allowed the discovery of additional *Listeria*-derived peptides that had previously neither been reported in the original article nor on the Immune Epitope Database (IEDB) adding to the list of targetable epitopes for this pathogenic bacterium. We demonstrate how reanalysis of previous immunopeptidomics data can add to the list of targetable epitopes and antigens with potentially high value in the battle against future viral pandemics and multidrug-resistant pathogenic bacteria.

Innovative aspects

- Software comparison for bacteria-derived immunopeptide identification
- Identification of novel *Listeria monocytogenes* epitopes

Factors of metronidazole resistance in *Trichomonas vaginalis*

Anna-Lena Mayr¹, Ana Paunkov², Karin Hummel¹, Ebrahim Razzazi-Fazeli¹, and David Leitsch²

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2) Institute of Specific Prophylaxis and Tropical Medicine (ISPTM), Medical University of Vienna, Vienna, Austria

Introduction

Trichomonas vaginalis (Tv) is a human parasite, causing urogenital tract infections, mainly in women. It is one of the most frequent sexually transmitted pathogens worldwide, responsible for approximately 156 million infections annually. [1] (WHO, 2016) Furthermore it can cause adverse pregnancy outcomes, and increase the risk of contracting HIV. [2] The parasite is most commonly treated with the antibiotic prodrug metronidazole, however resistance rates are on the rise. Therefore, it is crucial to find enzymes or other proteins involved in the process of resistance formation. In this study, proteome changes of different resistant and non-resistant clinical and laboratory strains were investigated, in order to determine differentially regulated proteins linked to resistance formation.

Methods

For cell culture different clinical and laboratory Tv strains were cultured in TYM medium. High levels of metronidazole resistance was introduced in strains C1 and T1 and compared to the respective antibiotic sensitive strain. Furthermore, strain C1 was depleted of iron using the iron chelator 2,2'-bipyridine, to test for iron dependence of enzymes when reducing and thus activating metronidazole. Different methods for protein extraction were tested and subsequently various protein digestion strategies were compared. These included filter-aided sample preparation (FASP), Single-pot solid-phase enhanced sample preparation (SP3) and Protifi S-Trap micro columns. In brief, FASP is a size exclusion method using an ultrafiltration unit available with different molecular weight cutoffs including 3 and 10 kDa. Proteins are reduced, alkylated, and digested on the filter, and peptides are then eluted. Similarly, S-Trap columns bind the protein to the matrix within the filter, and the same steps as with FASP are carried out. For SP3, carboxylate coated hydrophilic beads are used to bind the protein during reduction, alkylation and digestion. All samples are measured on a nano HPLC Ultimate 3000 RSLC system coupled to a high-resolution Q-Exactive HF Orbitrap mass spectrometer via an electrospray ionization (ESI) nano source interface.

Results and Discussion

Proteomic sample preparation using 3 kDa FASP filters provided the highest number of proteins, while also enabling to find small proteins like ferredoxins, believed to be important for metronidazole resistance formation in Tv. Bipyridil cultured Tv cells were found to have an almost identical proteomic profile as metronidazole resistant Tv cells, while remaining sensitive to the drug. In total, 53 proteins were found to be differentially expressed in both iron-depleted and metronidazole resistant cells of strain C1 as compared to sensitive ones. Furthermore, resistance induced lab cell lines (C1res, T1res) were compared to clinically resistant isolates (B7268, CDC085), and ferric iron reducing enzyme 1 (FR1) was found to be the only protein of known function that was strongly downregulated across all resistant cell lines. This enabled a proposal for a possible mechanism of how metronidazole resistance is formed in *Trichomonas vaginalis*.

Innovative aspects

- Protein digestion optimization for *Trichomonas vaginalis*
- Proteomic profiling of different clinical and laboratory *Trichomonas vaginalis* strains
- Relevance of potential protein candidate responsible for the formation of metronidazole resistance in *Trichomonas vaginalis*.

[1] Lamien-Meda, A., Leitsch, D. Identification of the NADH-oxidase gene in *Trichomonas vaginalis*. *Parasitol Res* 119, 683–686 (2020). <https://doi.org/10.1007/s00436-019-06572-8>

[2] Masha SC, Cools P, Sanders EJ, Vanechoutte M, Crucitti T (2019) *Trichomonas vaginalis* and HIV infection acquisition: a systematic review and meta-analysis. *Sex Transm Infect* 95:36–42

This research was funded in whole or in part by the Austrian Science Fund (FWF) [Grant DOI:10.55776/P35545].

Breaking Barriers in Crosslinking Mass Spectrometry: Enhanced Throughput and Sensitivity with the Orbitrap Astral Mass Analyzer

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Introduction

Crosslinking mass spectrometry (XL-MS) is essential for studying protein interactions in complex biological systems. However, XL-MS faces significant challenges in data acquisition and instrumentation. Complex datasets require sophisticated analysis, while low-abundance crosslinked peptides demand high sensitivity and accuracy during data acquisition. The dynamic range of mass spectrometers can miss both highly abundant and very low-abundance peptides, leading to incomplete data. Deep fractionation approaches or crosslink enrichment strategies attempt to overcome these dynamic range issues in current in-cell crosslinking workflows but come at the cost of long measurement times and demanding computational analysis. Time-consuming experiments can limit throughput and complicate large-scale analyses. Addressing these issues is crucial for fully leveraging XL-MS in protein interaction studies.

Results and Discussion

The Thermo Scientific Orbitrap Astral Mass Spectrometer revolutionizes XL-MS by offering rapid, comprehensive analysis with faster throughput, deeper proteome coverage, and higher sensitivity. Here, we present an optimized acquisition strategy for non-cleavable crosslinkers, delivering high-quality crosslinking data with drastically reduced sample input and shorter measurement times for high-throughput crosslinking experiments. Our comparison of the Astral mass analyzer with the Eclipse and TimsTOF HT instruments shows an increase in crosslink identifications by one-third or more for 100 ng sample input and with 70-minute active gradient time. This low sample input provides significant advantages for various crosslinking applications, such as crosslink-IP workflows, and is not limited to in-cell crosslinking experiments.

Investigating Dynamic Cellular Crosstalk and Pathomechanisms in Experimental Peritoneal Dialysis through secretomics analysis

Juan Manuel Sacnun¹, Klaus Kratochwill¹, Rebecca Herzog¹

¹) Medical University of Vienna

Introduction

Peritoneal Dialysis (PD) is a life-saving renal replacement therapy. Yet, the used of PD-fluids induce adverse effects leading to detrimental changes in the peritoneal membrane (PM), reducing the therapy efficacy. Cross-talk among different peritoneal cell types modulates PD-associated deterioration. Currently, there is no model available to study the interactions of these cells in close proximity. Here, we aimed to develop a co-culture model to explore cell-to-cell communication by analyzing the cellular proteome and secretome.

Methods

For modelling the PM, mesothelial and endothelial cells were co-cultured in transwell plates under optimized conditions for simultaneous culturing under non-starving conditions (5% FCS). Cells were exposed to PD-fluids in either co- or single-culture conditions. To overcome current limitation on secretomics analysis in non-starvation conditions, an equalizer approach was used to deplete high abundant proteins in combination with SILAC for tracing the cell origin of secreted proteins. For quantitative analysis of cellular and secreted protein abundances, LC-MS (TMT-18plex) was performed.

Results and Discussion

Co-cultured cells yielded differently regulated pathways following PD-fluid exposure compared to individual cultures. Analysis of the secretome revealed cell-specific pattern of secretion and low correlation with intra-cellular abundances. Combined proteome and secretome analysis revealed different ligand-receptor pairs expressed uniquely in co-culture. Furthermore, the secretome was able to capture different types of secretion. Using protein-protein interaction analysis, the identified cell-secreted proteins (~1900) formed 11 functional clusters, interacting with different receptors presented by the cells. The resulting interactome between cells through the secreted proteins in combination with differentially expressed cellular and secreted proteins revealed modulation of the cross-talk by PD-fluids. Identified secreted proteins had a high overlap with clinical samples from PD-patients and largely correlated with their clinical parameters. The combination of the modulation of the cross-talk by PD-fluids and clinical information revealed 3 novel candidates regulating pathways related to angiogenesis, EMT and NOTCH, which were validated in a chronic mouse model and patient samples. This study shows that harmful effects of PD-fluid exposure on mesothelial also affect endothelial cells. Co-cultures revealed different responses compared to individual cultures, highlighting the importance of models that allow interactions between multiple cell types. Secretomics analysis showed to be essential to understand cell-to-cell communication, and showed a more complex modulation than classical ligand-receptor interaction. We further identified novel potential signaling axes between the cell types explaining pathophysiological changes of the PM during PD that may allow identifying therapeutic targets to reduce current limitations of PD.

Innovative aspects

- First-of-its-kind study of a deep secretome in non starving condition (5%FCS).
- The secretome may trigger and/or regulate diverse pathophysiological processes through cellular cross-talk beyond known ligands.
- Three novel candidates could be validated in vitro, in vivo, and in patient samples, findings with implications for advancing understanding of cell-to-cell communication and improving our understanding of PD-induced pathophysiological changes of the peritoneum that limit duration of effective PD therapy.

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Effects of Alirocumab on Lipids and ApoB-Containing Lipoproteins Beyond LDL-C

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Introduction

Inhibition of Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) has become a therapeutic strategy for lowering cholesterol levels. The PACMAN-AMI (Effects of the PCSK9 Antibody Alirocumab on Coronary Atherosclerosis in Patients With Acute Myocardial Infarction) trial is a double-blind, placebo-controlled, randomised trial that evaluated the effect of Alirocumab on coronary atherosclerosis. The trial included patients presenting with acute myocardial infarction (AMI) who underwent percutaneous coronary intervention. Longitudinal plasma samples of 265 AMI patients who completed the 52-week follow-up were available for analysis. Building on our previous research employing apolipoprotein proteomics in patients with coronary heart disease (Clarke et al., *Circ Res* 2023), we assessed the impact of the PCSK9 inhibitor Alirocumab on apolipoproteins.

Methods

Untargeted and targeted mass spectrometry (MS) were used to quantify proteins and lipids. Discovery proteomics was performed by data-independent acquisition-MS. Parallel reaction monitoring-MS with stable-isotope labelled peptide standards (AQUA Ultimate, Thermo Scientific) was used to quantify 14 apolipoproteins in >1,000 samples. Lipidomic profiling was conducted using flow injection analysis-MS (Absolute IDQ p400 HR kit, Biocrates). Multiple testing in high-dimensional data was accounted for using the Benjamini-Hochberg procedure.

Results and Discussion

Discovery proteomics revealed an elevation in PCSK9 and a reduction in several apolipoproteins (ApoB, ApoE and ApoC apolipoproteins) in patients on Alirocumab. As early as 24 hours after treatment initiation, Alirocumab led to an increase in PCSK9 levels, with a median increase [interquartile range, %] of 262% [171, 378]. This was followed by an even more pronounced rise at 4 weeks and 52 weeks, reaching 1575% [1193, 2005] and 1480% [1067, 1814], respectively ($P < 0.001$ for all comparisons). The rapid increase in PCSK9 levels after 24 hours suggests immediate immunocomplex formation. A compensatory rise in PCSK9 production may contribute to later elevations. Alirocumab not only lowered LDL-C but also achieved sustained reductions in apolipoproteins associated with other ApoB-containing lipoproteins (ApoE, ApoC2, and ApoC3). After 4 weeks, the change in LDL-C was -85% [-91, -80] with Alirocumab vs. -52% [-60, -42] with placebo ($P < 0.001$). The corresponding change in ApoB levels was -73% [-78, -68] with Alirocumab vs. -42% [-49, -31] with placebo ($P < 0.001$, between groups). ApoE changed by -42% [-50, -29] with Alirocumab compared to -15% [-32, -3] with placebo ($P < 0.001$). Reductions in ApoC2 ($P = 0.0010$) and ApoC3 ($P = 0.0026$) were less pronounced. Notably, Apo(a) levels were unaltered in patients treated with Alirocumab compared to baseline. Lipidomics revealed pronounced reductions in cholesteryl esters, sphingomyelins, phosphatidylcholines, and diglycerides. While the former three are attributable to a decrease in LDL particles, the lower diglyceride levels suggest an impact on other intermediaries of lipid metabolism, such as intermediate-density lipoproteins and remnant lipoproteins derived from triglyceride-rich lipoproteins. These therapeutic effects are not reflected in the conventional measurements of total triglycerides.

Innovative aspects

- Unbiased exploration of the effects of PCSK9 monoclonal antibodies
- Combination of lipidomics and proteomics for a comprehensive analysis of lipoproteins

Discovery of a novel antifungal compound, ilicicolin K, through genetic activation of the ilicicolin biosynthetic pathway in *Trichoderma reesei*

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Introduction

In the quest to discover novel antifungal agents and new antifungal production processes, we investigated the biosynthetic gene cluster (BGC) for ilicicolin H in the fungus *Trichoderma reesei*. While the BGC is silent under standard cultivation conditions, we achieved to activate it by over-expressing its transcription factor TriliR.

Methods

Successful BGC activation was confirmed by RT-qPCR, proteomic and metabolomic analyses. Molecular networking led to the identification of related ilicicolin products.

Results and Discussion

Metabolomic profiling upon BGC expression revealed high-yield production of the supposed main product ilicicolin H. To elucidate the functionality of this BGC, we employed a combination of overexpression and deletions of individual biosynthetic gene cluster constituents. Deletion of *triliA*, encoding for the core polyketide synthase TriliA, completely ceased product formation, as expected. In contrast to previous heterologous expression experiments, we could demonstrate that the epimerase TriliE is necessary for the formation of ilicicolin H in the native host. While we hardly observed any of the previously reported side- or shunt products associated with heterologous ilicicolin H expression, we discovered a novel member of the ilicicolin family using a metabolomic molecular networking approach. This new compound, which we termed ilicicolin K, is expressed in substantial amounts in the genetically engineered *Trichoderma reesei*, enabling us to elucidate its structure by NMR. The structure of ilicicolin K is similar to that of ilicicolin H but differs by an additional hydroxylation and an intramolecular etherification of the hydroxyl group at the pyridine towards the tyrosine moiety of the molecule. Initial tests of ilicicolin K showed antifungal activity against *Saccharomyces cerevisiae* and *Aspergillus nidulans* with a similar minimum inhibitory concentration as ilicicolin H.

Innovative aspects

- High-yield expression of ilicicolin H by genetic BGC activation in a native host for the first time
- Discovery of a novel antifungal compound, ilicicolin K

Peptidomic analysis of cerebrospinal fluid samples reveals new biomarker candidates for amyotrophic lateral sclerosis

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Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating and progressive neurodegenerative disease that affects motor neurons and leading to death within 3 years. Diagnosis is based on clinical symptoms, the pathophysiology is still unclear for most patients and treatment options are limited. Therefore, there is an urgent need for fluid biomarkers to support basic research, diagnosis and drug development in ALS.

Methods

We applied mass-spectrometry-based peptidomic analysis in cerebrospinal fluid (CSF) samples of ALS and non-neurodegenerative control patients (Con) from a discovery (24 ALS, 24 Con) and validation cohort of patients (67 ALS, 42 Con). Peptides were isolated and purified from CSF for unbiased peptidomics in the discovery cohort and analyzed via mass spectrometry (MS). Selected peptide candidates were evaluated by targeted parallel reaction monitoring (PRM) in the validation cohort including stable isotope-labelled standard peptides. Analyses were performed with an UltiMate 3000 RSLCnano system and an Orbitrap Exploris 480 MS equipped with FAIMS interface. Screening data were processed with the PEAKS software and targeted PRM data were analyzed in Skyline. Peptide abundance changes in the validation cohort were correlated with clinical parameters.

Results and Discussion

We identified 33605 peptides in CSF samples from the discovery cohort and 56 of them were significantly regulated in ALS compared to controls. A coefficient of variation (CV) of 6.1% was obtained for the number of identified peptides over all patients and the abundance dynamic range of peptides in patient samples spanned eight orders of magnitude. Systematic selection for the best candidates revealed a targeted PRM method with 8 peptides, which was validated in terms of intraassay variation (CV 4.6-12.2%), dilution stability (accuracy 80-119.1%) and freeze-thaw stability (accuracy 88.6-148%). The targeted PRM confirmed the abundance changes of the discovery experiment for all peptides. Combination of all peptides in a logistic regression model led to an area under the curve value of 98% for the discrimination of ALS from controls. PRM data of the neurofilament light chain (NfL) peptide strongly correlated with an established NfL immunoassay (Ella) ($r = 0.968$, $n = 109$).

Innovative aspects

- We report eight peptides derived from seven proteins as biomarker candidates for ALS.
- The peptides are derived from proteins with different function and their determination with our PRM method provides the opportunity for simultaneous investigation of key processes in ALS and other neurodegenerative diseases (neurodegeneration, metabolic alteration, muscle weakness/atrophy and synaptic function or the secretory pathway).
- Future studies must clarify their clinical value for diagnosis, monitoring disease progression and treatment response, their changes in the preclinical phase of ALS and whether the peptide level changes are indicative of the parent protein concentration or reflect changes in protein processing.

Short Talk 9

Thoracic aortic diseases: Identification of diagnostic biomarkers using proteomic analysis

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Introduction

Thoracic aortic aneurysms (TAA) are common causes of death in western industrial nations. Diagnosis and risk assessment in patients with TAA remains challenging. Therefore, we aimed to identify possible circulating blood biomarkers for the diagnosis and prognostic assessment of thoracic aortic diseases by analyzing extracellular vesicles as well as human plasma samples by using mass spectrometry-based proteomics.

Methods

A total of 80 patients were divided into an aneurysm group (n=40) and a control group (n=40), and plasma samples were taken from each patient. To identify a potential biomarker for the diagnosis of TAA, plasma samples were analyzed using parallel reaction monitoring and extracellular vesicles extracted from plasma were analyzed using a bottom-up proteomics approach.

Results and Discussion

Overall, 1077 proteins were successfully identified in extracellular vesicle samples, including several well-known EV markers such as CD47, CD63, CD73, CD81 and FLOT1. In total, student's t-tests revealed 14 proteins to be significantly upregulated (p-value < 0.05, fold change > 1.5) in patient samples (i.e. Myeloperoxidase: p=0.03, fold change=2.0; Enolase 2: p=0.01, fold change=3.8) while 14 proteins were significantly upregulated in control samples. At the same time, PRM measurements show that the C-reactive Protein is significantly upregulated in patient samples. A range of potential TAA biomarkers were found among the significantly regulated proteins. For instance, Myeloperoxidase and Enolase 2 were both found to be associated with aortic disease in prior analyses and may therefore be valuable biomarkers for TAA. The identified biomarker candidates need to be validated in larger, external TAA cohorts before they can be used clinically for the diagnosis and prognostic assessment of TAA.

Innovative aspects

- Identification of potential TAA biomarkers from extracellular vesicles and human plasma

Less is More: Enhancing Glioblastoma Metabolomics by Minimizing Tissue Requirements

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Introduction

Glioblastomas are among the most malignant brain tumors and are characterized by their aggressiveness, low survival rates and high recurrence. One of the biggest challenges in diagnosis and treatment is the high tumoral heterogeneity often present. This aspect has been confirmed by imaging techniques, however, has often remained elusive in molecular analysis techniques, such as metabolomics based on mass spectrometry (MS). Routinely, clinical metabolomics is performed on a representative tissue sample, encompassing several milligrams, making it hard to analyze differences on cellular levels. The aim of this project was to elucidate the preparative and instrumental limits in metabolomics tissue analyses with the ultimate goal to miniaturize the analysis workflow for more in-depth investigation of the pathological mechanisms in glioblastoma.

Methods

Different amounts of homogenized glioblastoma tissue were extracted by means of the SIMPLEX protocol, allowing for simultaneous extraction of metabolites in 80% methanol and lipids in methyl-tert-butyl ether and analyzed on a dual HPLC system (HILIC, RP) coupled to a high-resolution orbitrap mass spectrometer. Data was acquired on MS² level for positive and negative polarities via data-dependent acquisition (DDA). Analysis was subsequently carried out in a quantitative, as well as non-targeted manner.

Results and Discussion

Detection of a set of key oncometabolites was achieved down to 0.05 mg sample extracted, albeit with limitations for experimental applicability above this threshold. Implications for quantification of a broad panel of hydrophilic metabolites and lipids will be discussed, as well as reproducibility, and number and quality of identifications in non-targeted data evaluation. Overall, the results indicate that MS-based metabolomics is suitable for the analysis of microscale sample amounts, such as obtained by punch-needle biopsies. These results could enable more insights into disease mechanisms via less invasive sampling methods.

Innovative aspects

- Absolute quantification of a large set of (onco-)metabolites and lipids within glioblastoma tissue
- Elucidation of preparative and instrumental limits in tissue metabolomics

Parchment – A Collagen Sample: MALDI MS, FTIR and Raman Imaging to Study Localised Proteome and Lipidome Changes

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Introduction

Proteomics tools play a crucial role in analyzing parchment, the primary writing material in Western Europe before the advent of paper. This writing material, despite its paper-thin appearance, was made mainly from calf, sheep, and goat skin. The labor-intensive production process reduces the skin to the dermis, resulting in a fibrous material composed mainly of a collagen network and lipids. Modifications and degradation of parchment are closely related to structural and molecular changes in the dermal material caused by handling and environmental stress. Oxidative stress, induced by light and atmospheric pollutants, accelerates lipid oxidation and collagen modifications, weakening the parchment's integrity and preservation. Traditionally, spectroscopic techniques have assessed damage at the protein level by observing changes in the collagen backbone. MALDI MS enhances the understanding of parchment degradation and the synergetic effects of individual analytes. Our study is the first to combine proteomic and lipidomic analyses with spectroscopic imaging to decipher degradation-induced changes in the parchment proteome and lipidome at the fibrillar level.

Methods

Parchment samples were SO₂- and light-aged under laboratory conditions. After OCT-embedding and vertical sectioning imaging experiments were performed on 8 µm cryo-sections using FTIR and Raman microscopy at spatial resolutions < 10 µm. Consecutive sections were analyzed by MALDI FTICR MSI (15 µm spatial resolution). Lipids were targeted after coating samples with 1,5 DAN via sublimation (home-built, patent pending). For protein imaging, samples were treated with COLase III and trypsin/LysC applied by spray-deposition (HTX TM5/Shimadzu iMLayer Aero), respectively, followed by incubation (37 °C, high humidity) and spray-coating with CHCA matrix. Peptide identity was confirmed by comparison against MS1 and MS2 results of robust in-solution digestion protocols. Data were analyzed/visualized with FlexImaging and SCiLS Lab software (Bruker).

Results and Discussion

Spectroscopic analyses assessed the disintegration of the collagen backbone (amide bands) and structurally important disulfide bonds. The delicate, highly collagenous material of thicknesses below 300 µm comprising fibrillar and gelatinized structures made sample preparation for MALDI MSI challenging. Nevertheless, OCT-embedding allowed the preparation of samples that could be used for lipid and peptide imaging. MALDI MSI allowed the identification and localization of sphingolipids, free fatty acids and different collagen types. Interestingly sphingomyels and fatty acids showed distinct localization on hair and flesh side of the parchment samples. The comparison of proteomic MSI results against in-solution digestion protocols allowed the identification of COL I (α-1(I) and α-2(I)) and less abundant collagen types, like COL V and VII. Preliminary results revealed characteristic mass shifts of 16 Da (oxidation) and 64 Da (SO₂-adduct) after artificial aging.

Innovative aspects

- first spatially resolved bottom-up proteomic and lipidomic study of parchment
- multi-modal imaging approach to study degradation effects induced by environmental factors

Integrating MALDI-MSI and imaging mass cytometry to reveal intratumoral heterogeneity in breast cancer subtypes induced by the tumor microenvironment

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Introduction

Breast cancer (BC) is a global health issue, affecting a high proportion of the female population. Spatial proteomics enables the study of the proteome within tissue context, offering a promising approach to unravel the biological processes contributing to BC heterogeneity that can lead to ineffective therapies. To study this heterogeneity, we use a combination of untargeted MALDI imaging (MSI), bulk tissue LC-MS/MS, and targeted imaging mass cytometry (IMC). This approach aids in identifying diagnostic and prognostic biomarkers masked by tissue heterogeneity and allows precise mapping and quantification of selected biomarkers. The co-registration of these techniques can enhance our ability to correlate molecular and spatial data, leading to comprehensive insights into tumor biology and potential therapeutic targets. We established an optimized protocol for sample preparation of gelatin and paraffin-embedded BC tissue for MALDI imaging, configured a high multiplex IMC-panel, and performed data integration to analyze BC subtypes.

Methods

Human BC cell lines (MCF-7, SKBR-3, MDA-MB-231) were inoculated into female athymic BALB/c-nude mice. Excised tumors were embedded in gelatin, paraffin, and liquid nitrogen. For MSI, we optimized pre-analytical and analytical parameters to enhance the signal-to-noise (S/N) ratio and enable protein identification. Following MSI, samples were stained for standard histological evaluation. Adjacent tissue sections were used for IMC. Proteins were also identified by TMT-based LC-MS/MS from homogenized tissue. Abstract text in total not more than 3500 characters including spaces.

Results and Discussion

Following the establishment of the MSI protocol, peptide signals were analyzed using spatial segmentation and uniform manifold approximation and projection (U-MAP) plots. Segmentation maps based on MSI aligned with expert-based histological assessment were generated. Unsupervised U-MAP plots enabled visualization of multidimensional information, allowing segmentation of different tumor, stroma, and necrotic tissue regions. Molecular BC subtypes were clearly distinguishable by MSI. In the bulk MS analysis ~17 000 proteins were identified, providing a source for confident matching of peptide signals from MSI. Based on the expression analysis of the TMT data and comprehensive literature review, we selected 28 markers for IMC. The targeted analysis revealed distinct marker expression patterns, with some markers predominantly expressed in specific subtypes. This detailed mapping highlighted unique proteomic landscapes across the tumor subtypes, including variations in pathways related to cell proliferation, apoptosis, hypoxia and immune response, thereby advancing our understanding of BC heterogeneity. Using the established workflow, peptide signatures not only differentiate histologically confirmed tumor regions but also reveal sub-regions that are indistinguishable by standard methods, demonstrating the potential of MSI as a tool for translational BC research.

Proteoforms - how do we get them intact out of tissues?

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Introduction

Proteoforms are the smallest unit of the proteome. The human organism is comprising more than 1 billion proteoforms coded by approximately 22.500 genes. Since different proteoforms coded by the same gene can have different molecular functions and are often associated with different biological processes, their identification and quantification matters, if we want to decipher molecular mechanisms in the organism. Very critical is the release of proteoforms of tissues during homogenization, since during this step compartments of cells are broken and enzymes released, which convert proteoforms in vitro, which will hamper the view on the original composition of proteoforms. Sampling of tissues with infrared laser systems is strongly reducing the risk of conversions of proteoforms.

Methods

Tissues are sampled with the picosecond infrared laser system (PIRL) by irradiating the surfaces of the tissues with the laser light. The resulting tissue aerosol, containing all molecules in a solubilized form, is condensed. The condensate is passed to sample preparation enabling the analysis of proteoforms (SDS-PAGE, 2D-electrophoresis, both followed by bottom-up proteomics; top-down mass spectrometry). The proteomics results of the samples obtained with tissue sampling and simultaneous homogenization with PIRL were compared with samples obtained from the same tissues and prepared with classical mechanical homogenization.

Results and Discussion

Tissue sampling and homogenization with PIRL is yielding a significant larger number of intact proteins in comparison to samples prepared by mechanical homogenization. This advantage of the laser sampling method can be explained by the very fast sampling and homogenization process guaranteeing minimal proteolysis. We therefore conclude, that tissue sampling with PIRL is giving access to the composition of proteoforms much more close to the original composition of proteoforms in tissues. In PIRL-samples the total yield of proteins was significant larger as well as the number of identified proteins compared to mechanical-homogenization samples. This advantage of PIRL-tissue-sampling is given by the complete and soft homogenization of the tissue, solubilizing a larger number and amount of proteins compared to mechanical homogenization. Condensed PIRL-induced tissue aerosols after centrifugation do not show any pellet after centrifugation in contrast to mechanical homogenization. In summary, sampling of tissues with PIRL is ideal for getting close to the original composition of proteoforms in intact tissues and for high yields of proteins concerning numbers and amounts.

Innovative aspects

- Soft and complete solubilization of tissues by irradiation with PIRL.
- PIRL tissue sampling is yielding samples with minimal proteolytic degradation of proteins.
- A minimal tissue ablation volume of 0.5 nl by a single laser shot is possible.

Targeted (Phospho)Proteomics Reveals mTOR Pathway Responses to Growth-Impacting Conditions in Zebrafish PAC2 Cells

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Introduction

Reversible protein phosphorylation represents a major mechanism in cellular signalling, including the mechanistic target of rapamycin (mTOR) pathway, which regulates growth and proliferation. Given its potential role in mediating the effects of toxic chemicals on growth, the mTOR pathway has recently drawn interest in ecotoxicological research. However, conventional antibody-based methods for studying protein phosphorylation are limited by costs, time, and frequent absence of suitable antibodies for non-mammalian models such as fish. To address these limitations, we developed a mass spectrometry-based targeted (phospho)proteomics method that allows simultaneous quantification of phosphorylation and abundance of multiple protein targets within the mTOR pathway in zebrafish (*Danio rerio*) PAC2 cell line.

Methods

Our optimized sample preparation workflow involves rapid cell lysis with 5% SDS and digestion using S-Trap™ columns, followed by NTA-Fe-based enrichment of phosphopeptides. Quantification assays were developed using synthetic peptides corresponding to the endogenous peptide targets representative of zebrafish proteins. Multiple Reaction Monitoring (MRM) on LC-MS/MS enabled a high detection sensitivity (median limit of quantification of 0.35 fmol/μL) and allowed monitoring of 17 mTOR pathway-related proteins. We applied this method to assess mTOR pathway responses under conditions known or expected to affect growth and proliferation of fish cells, including pharmacological inhibition of mTOR by Torin2, nutrient deprivation, and exposure to toxic chemicals, including a fungicide trifloxystrobin and an uncoupler 2,4-dinitrophenol (DNP). PAC2 cell cultures were treated in the exponential growth phase and samples were collected at 4, 24, 48, and 120 h post-exposure.

Results and Discussion

Torin2, while not acutely toxic, significantly inhibited cell growth upon chronic exposure and evoked strong changes in protein phosphorylation of several proteins (log₂ fold change of up to -5). All other chemical treatments also resulted in reduced cell proliferation after five days. Interestingly, while Torin2, trifloxystrobin and DNP caused differential time-dependent responses at upstream mTOR phosphosites, they all converged at similar effects at downstream mTOR phosphosites. The latter included down-regulation of sites related to mTOR complex activity, protein synthesis and mRNA translation. Nutrient starvation deactivated the mTOR pathway in a time-dependent manner, which was evident as early as 30 min after the medium change. Our study provided a time-resolved view of the mTOR pathway dynamics under different stressors and enabled identification of molecular markers, such as the phosphosite of PRAS40, which could potentially be applied to monitor the mTOR activity and help assess chemical impacts on fish growth.

Innovative aspects

- **Innovative LC-MS/MS Approach:** We developed a MS-based (phospho)proteomics method allowing simultaneous quantification of phosphorylation and abundance levels of multiple proteins in a zebrafish PAC2 cell line.
- **Optimized Workflow for Enhanced Sensitivity:** A workflow involving rapid cell lysis, S-Trap™ column digestion, NTA-Fe phosphopeptide enrichment and MRM-detection enabled achievement of high sensitivity and increased sample throughput.
- **Time-Resolved mTOR Pathway Analysis:** Obtained time-resolved insights into mTOR pathway dynamics under various stress conditions.

Novel microsampling approach based on SPME probes for monitoring the levels of endocannabinoids and phytocannabinoids in biological samples

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Introduction

The endogenous cannabinoid system is an important component of the organism that participates in multiple physiological processes, including energy metabolism, and motor activity. This system consists of several types of receptors, such as CB1, CB2, and ligands for those receptors, namely endogenous cannabinoids (endocannabinoids, ECs) [1]. Apart from ECs, those receptors are also affected by exogenous compounds, e.g. plant cannabinoids (phytocannabinoids, PCs). Solid phase microextraction (SPME) as a minimally invasive sample preparation technique facilitates isolation of low molecular weight compounds, such as ECs directly from biological matrix. We have already developed a methodology that enables direct monitoring of ECs in brain tissue with the use of SPME technology [2]. In this study, novel SPME devices (in-lab made blades and fibers) with C18 extraction phase are proposed as a novel strategy to monitor ECs, including anandamide (AEA), 2-arachidonoyl glycerol (2-AG), and N-arachidonoyl dopamine (NADA) as well as the PCs, namely cannabidiol (CBD) and its metabolite (7-COOH-CBD) in biological matrices, including serum samples.

Methods

In-lab made SPME probes (blades and fibers) were prepared according to the internal protocol. Optimization of the SPME procedure comprised the selection of extraction and desorption times of analytes from a mixture of serum and PBS solution (50:50, v/v), and tests of the extraction efficiency of SPME process. Analytical conditions of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) were optimized, and included the selection of an appropriate chromatographic column for the separation of analytes and the adjustment of the gradient elution program. Finally, analyzed compounds were separated on an ACE Excel C18-AR column (100 mm x 2.1 mm, 1.7 µm). The optimized methodology was applied for the extraction of ECs directly from various rat tissues under *in vivo* and *ex vivo* conditions.

Results and Discussion

Extraction of tested compounds was carried out for 30 minutes, and desorption from the C18 coating into the MeOH/IPA mixture (50:50, v/v) was carried out for 20 minutes. The optimized SPME-LC-MS/MS method enabled simultaneous isolation and analysis of all tested compounds and internal standards within 6 minutes. This method may enable monitoring of the levels of ECs as well as PCs and their metabolites in different rat tissues. Overall, the analysis of the endocannabinoid system after exposure to PCs may uncover the biochemical networks that may help to delineate diagnostic or therapeutic targets with the use of medicinal cannabis.

Innovative aspects

- It has been demonstrated that optimized SPME-LC-MS/MS method facilitates monitoring of ECs and PCs in real biological samples without the need for additional sample handling and/or tissue homogenization.

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Development of a new glycolipid spectral library for high-resolution mass spectrometry workflows

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Introduction

Gangliosides are a type of glycosphingolipid containing sialic acid, made up of a glycan and a ceramide component. These molecules are found on the surfaces of cells in almost all vertebrates and are crucial for cellular signaling and recognition. They are significantly involved in various diseases, such as cancer, Alzheimer's disease, and influenza.¹ Additionally, gangliosides are emerging as promising markers in the process of stem cell differentiation.² However, their analysis is highly challenging due to their complex structures and amphiphilic character. To date, confident annotations are limited by the lack of comprehensive standards, databases, and software tools. In this work we develop a glycolipid MS/MS library, focusing on gangliosides, for high-resolution mass spectrometry workflows.

Methods

The analysis of commercially available gangliosides and glycosphingolipid standards (GD1a, GD1b, GD2, GD3, GM1, GM3, GM4, GQ1b, Lactosylceramide and Ganglioside mixture, neutral Glycosphingolipid mixture, disialoganglioside mixture, and total ganglioside extract) was conducted using an LC-MS workflow. This workflow utilized a 30-minute reversed-phase gradient with isopropanol. Full MS and data-dependent acquisition (DDA) experiments were performed in both positive and negative ionization modes, employing either HCD fragmentation (Orbitrap IQ-X) or UVPD fragmentation (Fusion Lumos), guided by a ganglioside-specific inclusion list. The spectral library was generated using Compound Discoverer 3.3 and mzVault 2.3, followed by manual inspection using stringent quality criteria.

Results and Discussion

Spectral libraries for both positive and negative ionization mode have been developed, currently comprising around 80 entries across 10 glycolipid classes (GD1, GD2, GD3, GM1, GM2, GM3, GQ1, GT1, Gb3, and LacCer). In this ongoing project we aim to expand the library further and will soon make these resources available to the scientific community in an open-source format. By continuously enhancing these libraries and encouraging community contributions, we seek to broaden access to glycolipid research for comprehensive studies in the field.

Innovative aspects

- Developing a glycolipid database for high-resolution mass spectrometry workflow s
- First open source glycolipid MS/MS library
- Community access to comprehensive glycolipid assignment

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Activity-based tissue atlas of murine serine hydrolases in dependence of nutritional state

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Introduction

Serine hydrolases constitute a large and diverse family of enzymes that play pivotal roles in numerous physiological processes, including metabolism, signal transduction, and inflammation. These enzymes are characterized by a serine residue in their active site, which is crucial for their catalytic function. Serine hydrolases are involved in the hydrolysis of various substrates, making them essential for the regulation of lipid and carbohydrate metabolism, as well as protein processing. For instance, lipases, a subclass of serine hydrolases, are critical for the breakdown of triglycerides into free fatty acids and glycerol, which can then be used for energy production, especially during periods of fasting or increased energy demand. Other serine hydrolases contribute to lipid metabolism and the modulation of signaling pathways that govern energy balance. The regulation of serine hydrolase activity is highly tissue-specific, reflecting the distinct metabolic needs and functions of different organs. In the liver, serine hydrolases are involved in gluconeogenesis and the detoxification of xenobiotics, whereas in adipose tissue, they facilitate the release of stored fat. Muscle tissue relies on serine hydrolases for the rapid mobilization of energy substrates during physical activity, and the brain depends on these enzymes to maintain lipid homeostasis and neurotransmitter balance. This study aims to elucidate the effects of feeding state and dietary composition on serine hydrolase activity in key metabolic tissues of mice, providing insights into the dynamic and spatial regulation of energy mobilization and the potential for dietary interventions in metabolic health.

Methods

Fresh mouse tissues were incubated immediately after harvest with a serine hydrolase specific probe harboring a click moiety. This biorthogonal group was subsequently coupled to a biotin tag for enrichment of activity labeled enzymes on streptavidin beads. Enriched enzymes were subjected to label free mass spectrometry on a Bruker timsTOF operated in PASEF mode. To define a baseline of protein abundance, label free proteomics without enrichment was conducted additionally.

Results and Discussion

In this study, mice were subjected to different feeding regimens: fasting, regular feeding, and post fasting refeeding on either chow or high-fat diet. Samples from 12 individual tissues including liver, adipose tissue, brain, and muscle were collected and analyzed for serine hydrolase activities using activity-based protein profiling (ABPP). The results indicated significant variations in serine hydrolase activities depending on both the feeding state and diet. Fasting induced a marked increase in hydrolase activity in the liver and adipose tissues, while regular feeding maintained baseline activity levels. High-fat diet feeding resulted in altered activation of serine hydrolase activity dependent on fasting state. Surprisingly, even in brain we were able to pick up feeding dependent alterations in serine hydrolase activity. These findings underscore the dynamic and tissue dependent regulation of serine hydrolases by dietary inputs and highlight the potential for dietary interventions to modulate enzyme activity in specific tissues, with implications for metabolic diseases and therapeutic strategies.

Innovative aspects

- Feeding state dependent serine hydrolase activity profiles in 12 murine tissues
- Cross tissue analysis of diet and feeding state impact

Mapping of the human solute carrier transporter protein interaction landscape

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Introduction

The majority of chemical substances, like nutrients, vitamins, ions and drugs, require transporter to cross biological membranes and enter cells and organelles. Of the roughly 1,500 genes estimated to encode the human transportome, the largest group consists of the solute carrier (SLC) superfamily, with some 450 members. To ensure the homeostatic regulation of metabolism and energetic household, transporters need to act in concert and be linked to the regulatory processes and pathways of cells. While there is ample evidence that regulation of transporter action requires protein interaction, a comprehensive interactome of the whole human transproteome has not been described. We report here the first characterisation of the interactome of SLC transporters.

Methods

We applied an AP-MS protocol tailored to transmembrane proteins to map the protein interaction network for ~400 transporters. To process the roughly 1,800 data-dependent acquisition (DDA) measurements, we setup a pipeline around MSFragger and Philosopher. To predict protein interactions in a reproducible manner, we devised a machine learning-based method which integrates common PPI-scores (including SAINT, CompPASS) with several quantitative and uniqueness features. For validation, we assessed changes in protein levels and localization upon siRNA based genetic perturbations of identified interaction partners.

Results and Discussion

The generated interactome covers more than 18'000 novel protein interactions, providing the basis for understanding functional integration of this protein superfamily into the human interactome. Functional enrichment analysis revealed interaction partners throughout the life cycle of the transporters. Proteostatic regulation emerges prominently in this analysis. To streamline validation, we therefore focused on features common to all transporters, such as abundance and localisation. The functional validation campaign addressed 135 interactions, and in 40% of the cases, we could show that knock-down of the interactor caused changes in protein levels or localisation. Clustering based on PPI-network similarity allowed us to identify protein interaction which are shared between groups of SLCs. Co-purification analysis identified several protein complexes, which included the LIN-complex. Trafficking changes of SLC43A2 could be verified upon LIN-complex perturbation. For selected interactions we performed transporter activity assays to show a direct modulation of transporter activity upon perturbation of protein interactions. Our work provides thus important insight in the complex molecular network of SLCs and establishes an invaluable scientific resource.

Innovative aspects

- A systematic AP-MS analysis of interaction partners of 396 human transporters.
- SLC abundance and localization is regulated by many proteostatic interactions.
- Validation of SLC-interactors shows modulation of transporter activity.

Assay for quantitative and qualitative analysis of adsorption events to uncover loss of molecules during sample handling

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Introduction

The field of omics is progressing towards single-cell analysis, with sample sizes now approaching the detection limits of current mass spectrometry systems. A frequently overlooked factor contributing to reduced sample and result quality is the use of standard consumables like vials, tubes, and pipette tips during sample preparation. In most analytical applications, the loss of molecules is offset by using internal standards. However, in omics, where thousands of analytes are quantified simultaneously, applying an internal standard for each analyte is impractical. To address the issue of molecule adsorption to surfaces, optimizing parameters such as solvents, additives, and pH can be highly effective. This optimization is most successful when the nature of interactions with the surface in a defined solvent is well understood.

Methods

To quantify and characterize analyte loss due to adsorption, we developed an assay named “Adsorption Properties of Surfaces” (APS). APS utilizes a commercially available standard of tryptic HeLa peptides, which encompass a wide range of chemical properties. After incubation of the peptides with the surface of interest in a defined solvent, the tryptic peptides and a control group are analysed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and adsorption is quantified with software typically employed in differential quantitative proteomics. Our automated script-based analysis then identifies and characterizes the adsorbed peptides. APS can be adapted to various surfaces used in sample preparation and storage. Additionally, APS does not require knowledge of the surface chemistry, which is often unavailable due to patent restrictions.

Results and Discussion

With APS, we were able to quantify the loss of peptides to various surfaces used for sample preparation and storage and characterized the chemical interaction occurring between the analyte and the surface in an acidic solvent. In summary, APS is an LC-MS-based tool designed to precisely describe the adsorption characteristics of sample handling surfaces. This enables the minimization of analyte loss through the rational selection of parameters, which is particularly beneficial in scenarios where internal standards are not feasible, such as in omics studies and cases with very small sample sizes.

Innovative aspects

- Development of a testing system to perform high-throughput quantification of sample loss
- Detailed characterization of analyte and surface interaction
- Automated analysis facilitates the assay's usage

Diving deeper in the sea of tears: lessons learnt from the proteomic analysis of a highly variable body fluid

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Introduction

Different body fluids are considered as easily available possible sources of biomarkers for systemic and local diseases. Some “external” fluids (e.g., tears, urine and sweat) can be collected using non-invasive methods, while collection others (e.g., serum, plasma, cerebrospinal fluid/CSF) require methods with low or medium invasiveness. Proteins in these fluids are variable combinations from different sources, and methods, circumstances of sample collection and processing introduce additional levels variance. The “external” body fluids show generally higher intrapersonal variance in composition and are more affected by the collection method and environment. These variances can alter the abundance of different sub-populations of proteins, including highly abundant proteins in different ways, which can cause difficulties in application of normalization methods and may introduce some level of bias in quantitative results. In this work we have analyzed human tear samples collected using different methods and replicate samples collected at different time points. In addition to tear fluid, meibum, secreted by the meibomian gland were also analysed, to estimate its contribution to the tear proteome. Levels of variance were determined, and possible normalization and quality control (QC) methods were evaluated to decrease those. Identification of different proteome sub-populations was performed using different correlation, clustering and feature selection methods based on time and sampling method dependent variability.

Methods

Tear samples were collected using three different methods (capillary, Schirmer’s strip and the novel phenol red thread) from healthy subjects. Meibum samples were collected by Schirmer’s strips. LC-MS files of data independent analysis (DIA) were analysed using either predicted or sample specific empirical spectral libraries using DIA-NN. Identification of unexpected peptide modifications and nonspecific cleavages was performed by Fragpipe analysis of DDA data. Statistical data analysis was performed using different software packages both on protein and peptide level.

Results and Discussion

Based on correlation and detectability analysis we have identified clusters of proteins which were found to be mainly related to origin of them. Paired comparisons of meibum and tear fluid samples of healthy individuals helped the estimation of the contribution of meibomian gland to the tear proteome. We have identified personal differences in the level of nontryptic peptides and in some known or novel posttranslational modifications.

Innovative aspects

- Comparison of traditional and novel tear fluid sample collection methods
- Classification of tear proteins based on correlation and cluster analysis
- Identification and quantification of abundant peptidofoms without application of enrichment

Proteome-wide Non-Cleavable Crosslink Identification Using Sparse Matrix Multiplication with MS Annika 3.0

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Introduction

Cross-linking mass spectrometry has emerged as a prominent tool for the identification of protein-protein interactions and for gaining insights into the native structures of proteins. Over the last decades the field of cross-linking has seen continuous growth and the development of cleavable cross-linking reagents allowed studying systems up to human proteome-wide scale. However, while non-cleavable crosslinkers exert properties attractive for biological applications, their use always has been limited by computational data analysis tools not being able to handle the extremely large search spaces of non-cleavable cross-linking experiments. We here present MS Annika 3.0, an updated and improved version of our cross-linking search engine that efficiently tackles this so-called *n-squared* search space problem and allows identification of non-cleavable crosslinks beyond human proteome-wide scale.

Methods

Identifying crosslinks from non-cleavable reagents requires smart handling of the given protein database as the search space grows with its square, potentially yielding trillions of peptide candidate pairs to consider. In MS Annika every peptide and mass spectrum is encoded as a high-dimensional sparse vector and the whole protein database can therefore be represented as a large sparse matrix which allows efficient scoring of millions of candidates within a fraction of a second by multiplying this matrix with a spectrum vector. This super-fast algorithm is the core of the MS Annika non-cleavable search, identifying likely peptide candidates and significantly decreasing the search space. The top candidates are re-scored with our in-house developed peptide search engine MS Amanda and possible peptide pairs are combined to crosslink-spectrum-matches. Results are validated using a transparent target-decoy approach and can additionally be exported for more sophisticated validation with tools like xiFDR.

Results and Discussion

We compared MS Annika 3.0 to other commonly used cross-linking search engines and show that MS Annika is on par or better in terms of crosslink identifications while providing a more robust false discovery rate (FDR) estimation, reporting 75% less false positives than competing tools on average. Most importantly we could show that MS Annika is able to accurately identify more than 430 unique crosslinks at 1% estimated FDR from an experiment with *C. elegans* nuclei, using the full *C. elegans* proteome of over 26 000 proteins for search.

Innovative aspects

- Enabling non-cleavable cross-linking experiments up to proteome-wide scale
- Representation of peptides and mass spectra as sparse vectors and fast search based on these representations allowing scoring of millions of peptide candidates
- Extremely efficient search in both speed and memory, enabling searches being performed on normal office laptops

Investigating the cardioprotective effects of SGLT-2 inhibitors

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Introduction

Beyond their initial approval for treating diabetic conditions, Sodium-Glucose co-transporter 2 (SGLT-2) inhibitors have recently been found to also offer cardioprotective properties. Since cardiovascular problems are one of the major complications that diabetic patients face, this finding was groundbreaking. Meanwhile it was shown that the cardioprotective effects are not limited to diabetic patients and the inhibitors' approvals have been expanded respectively. Observations from clinical studies hint in the direction that those effects exceed the glucose lowering effect without offering an explanation for the underlying mechanisms. Since the drug's target protein SGLT-2 is exclusively expressed in the early proximal tubule where it is responsible for the reabsorption of glucose it remains inconclusive why the positive effects are observed in the heart.

Methods

To investigate what kind of an effect those drugs have on the proteome of cardiac cells, the human cardiac cell lines AC16 (cardiomyocytes derived from left ventricular heart tissue) and HCM (human cardiac myocytes) have been treated with Dapa-, Cana- and Empagliflozin in a physiological and a higher concentration. Besides classical proteomics also redox-proteomics and Glutathione as well as its dimer have been measured as marker for oxidative stress.

Results

Changes in Glutathione metabolism have been observed. This is among others reflected by changed ratios of reduced to oxidized Glutathione variants, as well as changes in abundance and oxidation state of proteins. Furthermore, proteomics data reveal upregulation of DNA replication and translation, additionally to changes in different mitochondria associated proteins.

Discussion

All drug treatments seem to strive to maintain oxidative metabolism. Furthermore the data indicate that observed effects on the proteome do not strongly correlate with the concentration level used for treatment.

Innovative aspects

- Solid dataset for cardiac cells having been treated with Gliflozins, incl. further aspects as oxidative stress

Posters

Identification of birch pollen protease substrates and their cleavage sites in human nasal epithelial cells by N-terminomics

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Introduction

Allergic rhinitis is caused by an immunoglobulin E (IgE) mediated immune response involving Th2-helper cells of the adaptive immune system and is defined as symptoms of sneezing, nasal pruritus, airflow obstruction nasal discharge. Today, it causes a major health burden among the population in industrialized countries, about 40 % of inhabitants of Europe and America are sensitized to date with an upward trend. Major allergens causing allergic rhinitis are contained in plant pollen. In Europe, the tree order of Fagales produces the most recognized pollen allergens, of which birch pollen is the most dominant one. The development of type allergies involves the innate as well as the adaptive immune system, with dendritic cells (DC) as intermediaries between both playing a key role in the development of an allergy in which an immune memory, involving allergen-specific IgE, develops. To date, three major mechanisms of initiating an allergen-specific immune response have been described: DCs sample the allergen and are activated by secondary signals by epithelial cells, or the epithelial cells are activated and recruit immunogenic DCs, or the allergen permeates through the epithelia. The secondary signal can be induced either via a toll like receptor (TLR)-agonist or protease activated receptor (PAR) stimulation on epithelial cells. Proteases, especially in pollen, have been shown to degrade tight junctions between epithelial cells and generate gaps in the protective layer and potentially stimulate PARs. Therefore, they represent a key target to study the mechanisms of sensitization.

Methods

The aim of this work is to identify pollen-protease substrates on the human nasal epithelium. Therefore mass-spectrometric (MS) techniques, called N-terminomics, will be used to analyze pollen-diffusate treated RPMI2650 cells, a model cell line for human nasal epithelium. The work will also include a viability analysis of the cells when treated with pollen, and a permeation assay. The viability analysis is performed to exclude proteolytic events caused by cell death from the data. The analysis of the paracellular permeability of cell-monolayers will validate the expected degradation of tight junctional proteins. Data from both assays will be used to optimize the incubation time of cells with pollen diffusate for the MS-experiments. N-terminomics is based on labelling all protein N-termini within a sample, including those generated by proteolysis (neo-N-termini). Afterwards, a tryptic digest is performed, and the tryptic peptides are depleted. By comparing the remaining N-termini of a degraded and a control sample, protease substrates can be identified. Additionally, by analyzing the position of the neo-N-termini within a protein sequence, the cleavage site sequences can be obtained.

The identification of the substrates will provide targets for further investigating the mechanisms of allergic rhinitis and potentially aid in developing anti-allergic drugs.

Results:

An increase in paracellular permeability of an RPMI2560 monolayer in response to birch pollen diffusate could be detected for the first 6h of treatment. The N-terminomics workflow is established and yields enrichment efficiencies of labeled peptides of above 97% and an enrichment of cleavage motives of 60% in a test run in which chymotrypsin treated samples were compared to a control.

An Integrative Multi-Omics Study on Horizontal Gene Transfer and the Impact of the Mega-Plasmid pCER270 in *Bacillus cereus* Group

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Introduction

The emergence of new pathogenic bacteria often results from horizontal gene transfer (HGT), leading to bacterial adaptation to new ecological niches. In the emetic lineage of *Bacillus cereus*, which is the causative agent of severe foodborne intoxications, the pXO1-like mega-plasmid pCER270 carries the structural genes *ces* responsible for the non-ribosomal biosynthesis of the emetic toxin cereulide. Adjacent to the *ces* operon, 222 coding sequences are predicted on pCER270, including genes for sporulation, germination, and transcriptional regulation. Our study aimed to investigate the impact of pCER270 transfer on pathogen emergence and environmental adaptation using an integrative multi-omics approach.

Methods

Using the emetic *B. cereus* reference strain F4810/72 as the donor, pCER270 was transferred via pXO16-assisted conjugation to other *B. cereus* group members, including psychrotolerant *Bacillus weihenstephanensis* and biopesticidal *Bacillus thuringiensis*. The transfer was confirmed by PCR targeting the *repX* (ori of replication) and *ces* genes, and both transconjugants and parent strains underwent genomics, transcriptomics, proteomics, lipidomics, and metabolic fingerprinting.

Results and Discussion

The results show that *B. thuringiensis* transconjugant produced similar cereulide toxin levels as the emetic donor strain, while *B. weihenstephanensis* transconjugants produced very low levels, indicating a significant impact of plasmid chromosomal crosstalk for bacterial adaptation and virulence. Moreover, the integrative analysis of the collected data provided a holistic view of the molecular traits influenced by the mega-plasmid at different omics levels, i.e., protein production, lipid composition, and metabolic pathways. These initial findings highlight the particular role of pCER270 in bacterial fitness, fostering the hypothesis that plasmids play a pivotal role in bacterial adaptation and pathogen emergence.

Innovative aspects

- By transferring the pCER270 plasmid from the emetic *B. cereus* strain to the other members of the group, such as *B. weihenstephanensis* and *B. thuringiensis*, the study shows how HGT can lead to significant physiological and virulence changes, which could be a driver for the emergence of new eco- and pathotypes.
- The applied multi-omics strategy allows for a detailed understanding of the impact of mega-plasmid pCER270 transfer at various molecular levels within the bacteria.

Addressing analytical challenges during metabolic profiling of sulforaphane in HepG2 cells

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Introduction

Sulforaphane is a phytochemical and belongs to the group of isothiocyanates. Various health-promoting effects for consumption of cruciferous vegetables or food supplements with high contents of sulforaphane are described, although little is known on structure-activity relationships. Cruciferous vegetables contain glucosinolates, which are converted to isothiocyanates by the myrosinase enzymes. Sulforaphane's glucosinolate precursor glucoraphanin occurs in broccoli especially in broccoli sprouts. As the sulforaphane mode of action in mammalian cells is not well understood, metabolic profiling is a useful tool to gain insights to the effects on the hepatoblastoma cell line HepG2. When performing such studies, the high reactivity of sulforaphane has to be considered and experimental modifications have to be made.

Methods

An aspect that requires modulation during the experimental workflow is the low stability of sulforaphane in cell culture medium. Sulforaphane was incubated in cell culture medium without cells for 24 h under cell culture conditions. As sulforaphane is described to bind to thiols, its stability was compared in cell culture medium with and without thiols. The analysis was performed using high performance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (HPLC-Q-TOF). For metabolic profiling HepG2 cells were incubated with the substances for 24 h. To quench the metabolism cold acetonitrile and water (4+1, v/v) containing phenylglycine as internal standard were added to the cells. Analysis was performed using an HPLC coupled to tandem mass spectrometry (HPLC-MS/MS).

Results and Discussion

Incubating sulforaphane in regular thiol-containing cell culture medium reduced its concentration by 50% of the initial concentration. Although thiol-free medium resulted in higher stability of sulforaphane (up to 70% after 24 h), the impact of the lack of thiols on the metabolome of HepG2 cells was considered to be too strong to use it for metabolic profiling. Therefore, regular cell culture medium was used. Finally, metabolic profiling data revealed that glycolysis, the tricarboxylic acid cycle (TCA) and the urea cycle are affected by sulforaphane. Effects on the amino acid levels in the cells were also observed. The alterations in the TCA and the amino acid levels can be explained by the antioxidative effect of sulforaphane. It is postulated, that sulforaphane induces the biosynthesis of glutathione (GSH), which results in lower cysteine, glutamine and glycine levels. The metabolic profiling data demonstrates lowered cysteine levels but hardly any decrease of glutamine levels. That could be linked to the effects on the TCA, because the data indicates a flux of the metabolites towards glutamine. Another mode of action described for sulforaphane is the impact on pathways generating NADPH, like pentose phosphate pathway. A flux toward this pathway is indicated by the observed alterations in the glycolysis.

Innovative aspects

- Low stability of highly nucleophilic compounds can be compensated by using thiol-free media.
- Thiol-free media causes strong metabolic alterations in HepG2 cells compared to complete cell culture media.
- Data regarding the effects of sulforaphane on the human metabolome were generated.

***In vivo* crosslinking by mass spectrometry using DSBSO and dual-enrichment methods to investigate the nuclear interactome**

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Introduction

Understanding protein-protein interactions (PPIs) is essential for elucidating cellular functions and mechanisms. Crosslinking mass spectrometry (XL-MS) has emerged as a vital tool for mapping PPIs within biological systems. However, the efficiency of XL-MS is often hampered by low yields of crosslinked peptides and high measurement times. This study aims to enhance the XL-MS workflow of K562 cells by employing the DSBSO crosslinker coupled with a dual-enrichment technique and without the time-consuming need to measure a multitude of sample fractions.

Methods

We applied the DSBSO in an *in vivo* context for whole cells and specifically the nucleus of K562 cells to prove the feasibility of in-cell crosslinking and enhance the detection of low-abundance protein-protein interactions by focusing on one compartment. This diverges from the common practice of using cell lysates or single proteins, providing a more comprehensive PPI analysis. Subsequent to crosslinking and lysis, we facilitated a column-based cleanup to remove non-bound crosslinker and other cellular debris, increasing the purity of the sample. We combined size exclusion chromatography (SEC) with bioorthogonal enrichment via click reaction using dibenzocyclooctyne (DBCO)-coupled magnetic beads, utilizing an optimized bead/protein ratio.

Results and Discussion

The optimized protocol led to a substantial increase in the detection rate of unique crosslinks. In K562 cells, an average of 2800 unique crosslinks/sample were identified with a false discovery rate (FDR) of 1%. During SEC, one specific fraction consistently contained approximately 80% (~2400 unique crosslinks) of all identified crosslinks, while the second highest fraction contained an average of 13% (~400 unique crosslinks). This high yield in fewer fractions significantly reduced measurement time by concentrating the crosslinks, allowing for more efficient analysis and higher sensitivity in detecting PPIs. These advancements hold promise for broad applications in biological research, including the study of disease-related PPIs and structural biology. Our study demonstrates the potential for revealing novel interaction partners and presents a robust and efficient platform for XL-MS.

Innovative aspects

- **Biorthogonal Enrichment:** Use of DBCO-coupled magnetic beads for enhanced crosslinked peptide detection.
- **Time Reduction by High Yield:** Significant increase in yield and sensitivity of crosslinked peptides, with reduced overall analysis time.
- **Potential Novel Interactors:** Identification of new interaction partners within the nuclear and whole cell proteome, enhancing the understanding of molecular pathways.

Benchmarking TimsTOF HT and Orbitrap Eclipse for Deep Proteome Analysis using DDA and DIA Workflows

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Introduction

Bottom-up proteomics is rapidly evolving with new instruments and methods demanding continuous evaluation. This study benchmarks the TimsTOF HT and the Orbitrap Eclipse mass spectrometer using three-proteome mixtures (yeast, E. coli, human) in known ratios analyzed with both data-dependent acquisition (DDA) and data-independent acquisition (DIA) workflows.

Methods

Mixtures of the three proteomes (total protein loads: 0-600 ng) were separated on ionopticks Aurora Ultimate columns and analyzed on TimsTOF HT and Orbitrap Eclipse in DDA and DIA modes. DDA data was processed with FragPipe, while DIA data utilized Biognosys™ Spectronaut™. Custom Python scripts (MsReport) were employed for computational analysis.

Results and Discussion

Analysis of three replicates per sample mixture in DIA mode on TimsTOF HT identified over 12,000 protein groups (7,800+ human, 2,000+ yeast, 700+ E. coli) and 130,000+ unique peptides, while over 11,000 proteins groups were identified with DDA on TimsTOF HT. Notably, >80% of quantified proteins had coefficient of variation (CV) below 10%, with a median CV less than 5%. Experimental protein ratios closely centered around the theoretical ratios. Very few E. coli proteins were quantified in the 0 ng E. coli sample, indicating a low false discovery rate (around 0.4%). DIA identified up to 19% more protein groups than DDA, comprising mostly low abundant proteins.

The established workflows offer deep proteome coverage with high accuracy and low false discovery rates, demonstrating their readiness for application to complex biological samples.

Innovative aspects

- Comparison of latest mass spectrometers for deep proteome analysis.
- Comparison of data-dependent acquisition (DDA) and data-independent acquisition (DIA) workflows.

Multidimensional positive pressure micro-solid phase extraction in proteomics research

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Introduction

Significant advances in proteomics in terms of speed, accuracy and precision of analysis have been achieved through high-throughput and sensitive technologies that allow in-depth analysis of complex protein mixtures in a relatively short time. Multidimensional liquid chromatography in conjunction with mass spectrometry is central to the bottom-up approach to proteomics as it increases sensitivity by reducing peptide complexity. Micro solid-phase extraction (μ SPE) is a simple and efficient sample preparation method for various instrumental and non-instrumental purposes.

Methods

The aim of the present study was to optimize and evaluate the orthogonality of multidimensional offline fractionation methods for different peptide sizes, polarities, GRAVY (GRand AVerage of hydrophobicity) and protein coverage index. To determine the combination of fractionation methods that provides the highest degree of independence, different stationary phases were evaluated using the μ SPE platform with positive pressure: Reversed Phase (RP), Sztong Cation Exchange (SCX), Hydrophilic-Lipophilic Balance (HLB), Quaternary MethylAmmonium (QMA) and Mixed Strong Anion Exchange/Reversed Phase (MAX).

Results and Discussion

The six fractions obtained from each fractionation were analyzed using an LC-MS/MS system. The results showed that for BSA peptides, for example, the highest orthogonality was achieved with a combination of hydrophilic-lipophilic and strong ion exchange chromatography stationary phases. Developed chromatography and mathematical model can be used for accurate single peptide fractionation prediction in complex peptide mixtures.

Innovative aspects

- The multidimensional bottom-up fractionation of tryptic peptide samples is completed in max. 45 min and is fully automated
- Clean-up and 2-D fractionation can be predictive with regard to specific peptide elution
- Versatile stationary phases could be mixed and optimized in a controlled manner in the process of separating 2-D peptide mixtures

Is Robotics truly Superior? Reproducibility and hands-on Time of Manual and Automated in-gel Digestion

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Background/Objective:

Current technologies in proteomics enable the analysis of thousands of proteins from less than a microgram of protein extract. Quality and reproducibility are highly dependent on preanalytics. Despite technological advances many researchers still rely on manual sample processing and limited data on manual vs automated preanalytics is available.

Objective

Aim of our ongoing study is to quantify and compare systematically predefined quality checkpoints for in-gel digestion of complex protein samples for proteomics.

Results and Discussion

Processing time ranged from about 5 to 8 hours for 12 samples (protocol with 26 steps) depending on experience. Without experience, volunteers required 413 ± 40 minutes vs. 347 ± 15 mins on a fifth day of training ($n \geq 7$). Experimenters with more experience were only marginally faster than the average volunteer with training. In comparison, automated in-gel digestion required only slightly more than one hour for all manual steps. Notably, increasing the number of samples by 4 added about one hour of processing time for manual in-gel digestion whereas it had a negligible impact on automation. Transfer of sticky gel pieces to a microplate compatible with automation had a larger impact. During manual processing, 2 % of the samples were lost and 23 % were affected by deviations of the digestion protocol (630 samples). The deviations were further categorized into major (likely affecting sample quality: 2 %) and minor (appearing correctable: 21 %). Up to 25 % of one batch of samples were lost and 42 % affected by major deviations. Unexpectedly, sample loss and protocol deviations did not correlate with the number of training days. Precise calibration and systematic optimization reduced the number of lost samples during automated processing to 3 % and eliminated deviations. LC-MS/MS analysis showed that manual vs. automated digestion (>1 year experience in both) of a human cell extract resulted in 3.5 % more protein groups (mean: 4668 vs. 4505, 1 % FDR, approx. 300 ng on column, $n=10$) at similar CVs (1.5 % vs 1.7 %). Automated digestion resulted in less missed cleavages though (e.g. 76.1 % vs. 73.5 % without missed cleavage) at lower CVs (e.g. 2.5 % vs. 4.1 %). Whether minor deviations of the digestion protocol are truly correctable is currently under investigation.

Innovative aspects

- Processing time between experimenters varied by up to three hours with negligible walk-away time. In contrast, automation provided reliable overall processing time and required as little as approx. one hour hands-on time.
- A slightly higher number of protein identifications after manual digestion was associated with an increase in missed cleavages and larger CVs.
- Overall, automated in-gel digestion is more reliable and consistent in terms of intra- and interday reproducibility.

Proteo-Metabo-Flux – Linking Acetyl-CoA and acetylation dynamics for accurate determination of histone acetylation rates

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Introduction

Histone acetylation is an important post-translational modification that regulates DNA accessibility and gene translation. The substrate for this modification is the small molecule acetyl-CoA, which plays a central role in carbon metabolism. To fully understand the biological function of this reversible post-translational modification it is not sufficient to determine static acetylation levels but it is necessary to capture individual acetylation and deacetylation reaction rates. We have developed a quantitative proteo-metabo-flux approach and pipeline including the simultaneous extraction of acetyl-CoA and histones from cell culture as well as normalisation and correction algorithms to quantify site-specific histone acetylation rates and to combine them with acetyl-CoA turnover dynamics.

Methods

Cells were metabolically labelled with [U-13]-C glucose followed by a simultaneous extraction of acetyl-CoA and histones at different time points [1]. Histones were chemically acetylated with ¹³C₄,D₆-acetic anhydride and digested using trypsin to obtain chemically equivalent peptides [2]. Purified acetyl-CoA was measured with HILIC-SIM-MS (Orbitrap Fusion Lumos). Histone peptides were analysed with nanoLC-MS/MS (Orbitrap Fusion Lumos). Ordinary differential equations were used to build an integrated model of label incorporation into both acetyl-CoA and histone acetyl groups.

Results and Discussion

Here, we present our Proteo-Metabo-Flux-Quant (PMF-Quant) pipeline for the analysis of acetyl-CoA and histone acetylation dynamics.

We find that ¹³C from glucose is incorporated into the acetyl-, ribose- and adenine moieties of acetyl-CoA over a 6 hour period, with label incorporation plateauing at approx. 70%. Taking this partial incorporation into account is important when calculating histone acetylation rates, which are otherwise greatly underestimated. As proof of principle, we treat cells with the Akt inhibitor MK-2206, which inhibits glycolysis. This leads to an apparent change in histone acetylation rates when the correction is omitted, which is shown to be an artefact of upstream effects.

Innovative aspects

- Combination of metabolic and chemical labelling with simultaneous extraction of acetyl-CoA and histones.
- Use of ordinary differential equations to determine histone acetylation rates, combining metabolite and PTM-level information

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Decoding ECM Complexity in Scaffold-Free Spheroids: Proteomic Insights into Chondrogenic Differentiation

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Introduction:

Tissue engineering (TE) offers approaches for treating degenerative bone diseases and enhancing implantology. While traditional 2D cell cultures have been extensively studied, scaffold-free 3D spheroid cultures, which more accurately mimic native tissue environments, remain less explored. This study leverages human adipose-derived stem cells (ASCs) to generate scaffold-free spheroids for chondrogenic differentiation, aiming to provide advanced *in vitro* models for bone disease research, graft development and alike. While conventional techniques such as RT-qPCR and Western blotting provide valuable insights, they lack comprehensive coverage of protein dynamics. Liquid chromatography-mass spectrometry (LC-MS) based Proteomics, offers a broader and deeper view of the proteome. In this study we present our first results on scaffold-free 3D cell culture including proteomics data to enhance our understanding of protein-level changes crucial for chondrogenic differentiation.

Methods

TERT immortalized ASCs were cultured to form scaffold-free spheroids (2.5×10^5 cells/spheroid), which were then differentiated in chondrogenic medium over 28 days. Differentiation was validated using Alcian blue and immunohistochemical staining (IHC) for collagen types I and II. Gene expression of chondrogenic markers was assessed by RT-qPCR. For proteomic analysis, proteins were isolated from minced spheroids, and digested with collagenase III and trypsin/LysC following reduction and alkylation of proteins. Samples were analyzed on a Synapt-G2-HD mass spectrometer following peptide separation on a Hitachi LaChrome Elite HPLC system. Protein identification and quantification were performed using PLGS, IsoQuant and Perseus v2.1.0.0.

Results and Discussion:

RT-qPCR analysis confirmed successful chondrogenic differentiation, evidenced by significant upregulation of collagen types I, II, and X by day 21, alongside elevated expression of proteoglycan genes such as ACAN. The master regulator SOX9 was consistently upregulated throughout the experiment, while the antagonistic regulator Runx2 remained suppressed. Alcian Blue staining demonstrated substantial proteoglycan deposition by day 28, with notable staining observed across the spheroid cross-section. IHC revealed that collagen deposition primarily occurred in the outer layers of the pellets, with COL2 forming a distinctive ring-like pattern. Proteomic analysis further validated these findings by identifying multiple collagens and key matrix proteins, including decorin, biglycan, vitronectin, laminin, and fibronectin, within the spheroids. The protein profile highlights extensive ECM development and confirms effective chondrogenic differentiation. These results underscore the value of our proteomics approach in revealing detailed ECM components and understanding the intricate process of cartilage formation.

Innovative Aspects:

- Chondrogenic differentiation in 3D spheroids
- ECM proteome profiling in 3D tissue constructs
- Spatial analysis using Histology, IHC and MALDI-IHC MS Imaging

Proteome Analysis of Tissue Samples in Patients with Crohn's Disease

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Introduction

Crohn's disease is a type of inflammatory bowel disease (IBD) which may be activated by a disturbance in the intestinal barrier or variations in the composition of the gut microbiome, known as dysbiosis. This disease typically causes inflammatory patterns which may affect different areas of the digestive tract. In most cases, the colon and terminal ileum are affected, with the inflammation occurring transmural or in skip lesions. Symptoms from patients suffering from Crohn's disease vary, but can include severe abdominal pain, diarrhoea, rectal bleeding, and eventually weight loss. These symptoms negatively impact the patient's quality of life and may even account for various life-threatening complications [1–3]. Therapeutical treatments usually target patient's symptoms, as the underlying pathomechanism of the disease remains unclear.

Methods

The proteomic analysis of healthy and inflamed tissue samples collected during surgery and a routine colonoscopy was investigated to elucidate the molecular pathways involved in Crohn's disease. These samples were dissolved with an ultrasonic stick, digested, and then analysed with a timsTOF Pro mass spectrometer to determine the proteome profiles of the inflamed regions. Trapped ion mobility spectrometry (tims) – time of flight (timsTOF) mass spectrometry is a combined ion mobility mass spectrometer with an ultra-high-resolution time of flight (TOF) mass analyser, where separation occurs according to the mobility of an ion through a gas stream in an electric field [4, 5].

Results and Discussion

Proteome profiling comparing healthy and inflamed tissue was performed. As expected, proteins involved in inflammatory responses were upregulated in comparison to healthy tissue samples. Furthermore, hypoxia-induced protein levels also rose, potentially through the limited oxygen supply deriving from inflammatory mediated vasoconstriction. Another protein class regulated by oxygen supply; mitochondrial proteins were found downregulated. This may have led to a mitochondrial dysfunction through increased mitophagy, resulting in limited ATP production. Finally, the limiting ATP may result in lower levels of antimicrobial and metabolic proteins, which could be major causes of the disease conditions.

Innovative aspects

- Patients' proteome reveals that inflammatory responses may induce vasoconstriction, hypoxia, mitochondrial dysfunction, and finally, a lack of antimicrobial defence and detoxification capacity, which could play a role in the pathomechanism of Crohn's disease.

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Tryptic digestion on a budget - Opportunities and potential limitations of self-modified trypsin

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Introduction

In bottom-up proteomics methods, the use of a proteolytic enzyme is crucial. The most commonly used protease is high-quality sequencing-grade trypsin, which is approximately 40-fold more expensive than trypsin used for other applications.

Therefore, experiments were conducted to improve the quality of inexpensive trypsin products formerly made for routine cell culture applications. To increase the stability of trypsin, its resistance against autodigestion was improved by a methylation of amino acid side chains (which are partly targets of trypsin for digestion). Chymotrypsin is a less specific protease with different affinity to certain substrates, which commonly contaminates crude trypsin products. By using affinity chromatography, trypsin products can be further purified.

Methods

In this work trypsin was subjected to reductive methylation using formaldehyde and 2-picoline borane complex. Affinity chromatography was employed using *p*-aminobenzamidine agarose as a stationary phase. Several parameters were varied to determine optimal conditions for the most effective trypsin enhancement. The quality of the products was tested by autodigestion screening as well as test digestions of different protein samples. All self-made trypsin samples were compared with sequencing or higher grade trypsin for bottom-up proteomic applications. The analyses were performed by high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS) in data dependent acquisition mode. The data were processed using MaxQuant to determine peptide quantities and to evaluate contaminating proteases.

Results and Discussion

The developed protocol can be used to refine low-quality low-cost cell culture grade trypsin for bottom-up proteomic sample preparation. Following reductive methylation and purification, the yielded trypsin has very similar activity compared to commercially available sequencing-grade trypsin. Because of the very low cost of the self-modified trypsin, it may routinely be applied in high trypsin:substrate ratios, which decrease the required time for digestion and consequently, the overall sample preparation time. Several sample preparation techniques including peptide enrichment approaches and fractionation methods demand higher initial protein amounts and therefore require significant amounts of trypsin. The availability of inexpensive, high-quality trypsin makes these techniques more attractive for routine applications.

Innovative aspects

- Low-cost trypsin makes digesting proteins with higher amounts of peptidases for routine fast sample preparation more attractive
- Upscaling of proteolytic digestions becomes less expensive, reducing the costs of pre-fractionation

Designing an XL-MS/MS approach to investigate endogenous BORG protein assemblies

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Introduction

Lysosomes not only mediate cellular clearance but are also involved in various crucial processes such as combating microbes, triggering apoptosis, releasing exosomes, and signaling. Some of these functions rely on the positioning of lysosomes within the cell. The BLOC-1-related complex (BORG), composed of eight subunits, regulates the movement of lysosomes along microtubules by recruiting ADP-ribosylation factor-like protein 8B (Arl8b). We have shown previously that the BORG subunit Lyspersin is the linker between the BORG and the late endosomal/lysosomal adaptor and MAPK and mTOR activator (LAMTOR, Ragulator) complex using chemical cross-linking. The LAMTOR complex functions as a negative regulator for the BORG directed lysosomal transport.

Understanding molecular mechanisms of the signaling pathways mediated by lysosomes remains a challenge. Therefore, unraveling the subunit topology of BORG protein complexes and identifying the specific protein-protein interactions involved will aid in bridging these gaps in knowledge.

Methods

We aim to elucidate BORG protein assemblies under diverse physiological conditions, thereby enhancing our understanding of lysosomal signaling. Therefore we employ cross-linking mass spectrometry on endogenously tagged BORG subunits, such as N-ALFA-tagged Diaskedin.

The native BORG assemblies are isolated by affinity purification and immediately subjected to cross-linking, or they are purified from cross-linked lysosomes to identify organelle-specific protein assemblies. The purified protein-complexes are identified by LC-MS/MS and different database search engines will be utilized for data analysis.

Results and Discussion

The generation of the endogenously tagged BORG subunit was achieved by CRISPR/Cas9 ribonucleoprotein-mediated knockin in hTERT-RPE-1 cells.

For clonal isolation, we performed single-cell cloning by limiting dilution, followed by screening *via* polymerase chain reaction and validation by Western blot analysis.

We developed a workflow to purify endogenous BORG and interacting proteins *via* knockin Diaskedin using ALFA-specific nanobody immobilized agarose-magnetic beads. Currently we optimize the procedure to ensure a high quality and specificity of purified protein complexes.

Innovative aspects

- Endogenous knockin of ALFA-tagged Diaskedin in hTERT RPE-1 cells *via* CRISPR-Cas9
- Purification of native protein assemblies from cross-linked lysosomes and cell lysates with anti-ALFA magnetic beads
- Comparing different cross-linkers for a comprehensive XL-MS/MS analysis of endogenous protein complexes

Time-Resolved Mass Spectrometry-Based Multi-Omics Characterisation of the NISTCHO Bioprocess

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Introduction

To meet the growing demand for recombinant biotherapeutics in use today, producer cell lines are under constant development to improve their productivity, stability and product quality. Enhancing our understanding of biological responses during cultivation through host-cell omics, complementing conventional bioprocess monitoring and medium-based measurements as proxies for cellular function, may enable immediate adaptation of critical process parameters (CPPs) to ensure efficient and quality-assured production.

Methods

The design and evaluation of a fast, feasible and representative multi-omics sampling and downstream analysis approach for suspension cultures utilising state-of-the-art methodology and instrumentation were conducted. The resulting workflow allows for analysis of the host-cell proteome, phosphoproteome, metabolome, and secretome.

To demonstrate applicability, a fed-batch culture of the openly available, monoclonal antibody-producing cell line NISTCHO was consequently examined for the impact of feeding on cellular processes as indicated by changes in the proteome and reversible phosphorylation of proteins. Sampling was performed 60 min prior to feeding, as well as 15, 60, 90, 120 and 240 min post-feed, in both exponential and stationary growth phases to reflect multiple stages of the bioprocess. Parallel monitoring of nutrient consumption and metabolic excretions was deployed. To fully leverage the high-resolution LC-MS/MS data generated on the Bruker timsTOF pro, curation of the Chinese hamster (*Cricetulus griseus*) reference proteome, reducing redundancy and improving annotation and coverage, was performed. Additionally, to utilise the statistical power of time-course data, an in-house R package was developed to identify and cluster significant temporal dynamics of features.

Results and Discussion

A bioprocess-integrated multi-omics sampling and analysis workflow, addressing multiple key challenges, was designed and implemented. Further evaluation included investigation of a NISTCHO fed-batch culture. This work provides comprehensive temporal profiling of protein and protein phosphorylation dynamics in response to changes in nutrient availability around feeding windows distinct to different phases of the bioprocess. Key results range from master regulators of redox homeostasis and metabolism to pinpointing individual regulatory sites of DNA repair enzymes.

We emphasise that mass spectrometry can provide an in-depth analysis of cellular function in various product and process-relevant biological settings. The advanced characterisation of an academically available producer cell line and the generation of high-quality bioprocess reference data as part of our DigiTherapeutX project will fuel research efforts towards integrated digitalised production and predictable quality of protein therapeutics.

Innovative aspects

- Design and implementation of a bioprocess-integrated multi-omics sampling and analysis workflow
- Data processing and analysis pipeline includes curated *Cricetulus griseus* reference proteome and in-house software to handle time-course data
- Comprehensive temporal profiling of more than 7000 proteins and 3000 phosphorylation sites in response to feeding distinct to different phases of the NISTCHO fed-batch bioprocess

Transforming MS Data into Publication-Ready Excel Reports with XlsxReport

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Introduction

XlsxReport is a Python library developed by the Mass Spectrometry Facility at the Max Perutz Labs specifically designed to simplify the creation of well-formatted Excel reports from quantitative mass spectrometry (MS) results. The primary motivation behind developing XlsxReport was to address the repetitive and time-consuming task of manually formatting MS data into Excel reports. By automating this process, XlsxReport enables the creation of consistent and publication-ready Excel reports with minimal effort.

Methods

XlsxReport is an open-source project published under the Apache 2.0 license, it is accessible on GitHub and can be installed via PyPi (the Python Package Index). The project is actively maintained and utilizes continuous integration (CI) for automated testing to ensure reliability. Adhering to high standards of code quality, XlsxReport is designed for easy maintainability and expandability.

Results and Discussion

A frequent requirement when conducting MS analyses is creating Excel reports for manually inspecting results, sharing findings with collaborators, and including supplementary tables in scientific publications. XlsxReport addresses this need by utilizing reusable YAML-based template files that define the arrangement and formatting of tabular data for the creation of Excel files.

These templates allow users to choose which columns to include, determine their order, and organize columns into sections. Furthermore, Excel formats and conditional formats can be specified and applied to individual columns and sections, offering a high degree of customization for the report. Specific templates can be created for each data analysis pipeline and existing templates can be easily adapted for other pipelines, providing a versatile solution for generating uniform reports across different workflows.

XlsxReport offers a command-line tool for applying YAML templates to individual CSV files of MS results, streamlining the creation of Excel reports. Beyond the command-line tool, XlsxReport provides a rich Python interface for working with YAML templates and creating Excel reports. This interface is particularly useful in interactive environments like Jupyter notebooks, where the Python representation of a YAML template file allows for easy inspection and modification of existing templates. Additionally, a report builder class enables the creation of multi-sheet Excel files and the dynamic adaptation of templates to individual datasets. This extends the command-line tool's capabilities, enabling more complex workflows and offering users greater flexibility in report generation.

By utilizing reusable YAML templates XlsxReport streamlines the process of creating well-formatted and consistent Excel reports from quantitative MS results. Its user-friendly command-line interface and versatile Python interface make it a valuable resource for the mass spectrometry community, regardless of the user's Python expertise.

Disrupted Lipid Catabolism in Lung Cancer: Insights from Activity-Based Proteomics and Lipidomics

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Introduction

Lipid metabolism is increasingly recognized as a critical factor in cancer progression, with lipid droplet accumulation emerging as a hallmark of aggressive cancers. However, the role of lipid hydrolysis—specifically the breakdown of stored lipids—in lung cancer remains underexplored. In this study, we aimed to investigate the involvement of lipid hydrolysis enzymes, particularly adipose triglyceride lipase (ATGL) and monoglyceride lipase (MGL), in non-small cell lung cancer (NSCLC).

Methods

Our initial investigations involved a comprehensive analysis of tumor and adjacent normal tissue from NSCLC patients using activity-based protein profiling (ABPP), shotgun proteomics, and lipidomics. Building on these findings, we explored the functional consequences of lipid hydrolase depletion by generating knockout models of ATGL and MGL in a panel of NSCLC cell lines.

Results and Discussion

We found significant downregulation of several lipid hydrolases, including MGL, in lung tumors compared to normal tissue. Lipidomic profiling revealed substantial triglyceride accumulation and elevated levels of ceramides and lysophosphatidylcholines in tumors, suggesting a disruption in lipid catabolism. Interestingly, we observed that the deletion of ATGL or MGL in NSCLC cell lines led to increased proliferation in some but not all cell lines, which correlated with elevated de-novo fatty acid synthesis. These results suggest that the downregulation of lipid hydrolases may contribute to cancer cell proliferation by altering lipid metabolism.

To further elucidate these mechanisms, ongoing experiments are investigating nutrient utilization, lactate excretion, as well as mitochondrial respiration and glycolysis. Additionally, we are generating cell lines overexpressing lipid hydrolases that were found to be downregulated in tumors to assess reversibility of the observed metabolic changes and its effect on proliferation.

Overall, this study underscores the significant role of lipid hydrolysis in lung cancer metabolism and progression.

Innovative aspects

- Direct measurement of lipid hydrolase activity in freshly excised lung tumors and paired normal tissues, providing immediate insights into enzyme functionality
- Comprehensive combination of activity-based proteomics, shotgun proteomics, and lipidomics to create a holistic profile of lipid metabolism dysregulation in lung cancer
- Application of cutting-edge mass spectrometry techniques to identify novel metabolic biomarkers and therapeutic targets directly from patient-derived samples

Synthetic CDR3 derived from nanobody retains binding ability to *Borrelia*

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Introduction

Nanobodies (NB) represent a new type of biological substance that could be used in nanomedicine to treat a variety of human diseases, including cancer, neurodegenerative diseases, and infections. They are derived from the variable region of the heavy chain only antibodies naturally occurred in the *Camelidae* family and retain the full binding capacity as conventional antibodies. They are distinguished by good penetration in difficult-to-reach tissues, stability under high pressure (500-750 MPa) and extreme pH (3-9), resistance to proteolytic degradation, ease of modification, and recombinant production in express systems. NB are useful therapeutic tools as they can be incorporated into drug delivery systems by conjugating them on nanocarriers. NB consist of four conserved framework regions (FR 1-4), and three hypervariable complementary determining regions (CDR 1-3). CDR3 is the primary contributor to antigen binding (60-80%). CDR3 may have a long protruding shape and a convex paratope, allowing easier access to binding pockets inaccessible to conventional antibodies. In this study, we aimed to develop NB against *Borrelia bavariensis* using phage display. The CDR3 from best NB that binds *Borrelia* was sequenced and produced by solid phage peptide synthesis. The synthetic CDR3 was tested for its binding ability to *Borrelia*. Our next goal is to develop a drug delivery nanosystem targeting *Borrelia* that will have potential for the treatment of *Borrelia* pathogenesis. Research was funded from APVV-22-0084, VEGA1/0381/23, VEGA1/0348/22, EURONANOMED2021-105.

Methods

Llama was immunized with inactivated *Borrelia bavariensis*. One week after the last immunization, the blood was collected and peripheral blood mononuclear cells were isolated. RNA was extracted and reverse transcribed to generate a NB-*E.coli* library. Phage display involving biopannings using fixed *Borrelia* was performed to generate NB-phage library. Gene coding NB was amplified from eluted phages, cloned into to *E. coli* SHuffle T7 expression system and soluble NB were produced. The NB were purified by nickel affinity chromatography. The production was confirmed by lithium dodecyl sulfate-polyacrylamide gel electrophoresis. NB size and purity were confirmed by matrix-assisted laser desorption/ionization mass spectrometry. Affinity of purified NB to fixed *Borrelia* was tested by ELISA. The NB clone with the highest interaction was sequenced to identify the CDR3 sequence that was used for commercial production. The ability of NB and CDR3 to interact with fixed *Borrelia* was assessed by ELISA.

Results and Discussion

From pannings, the NB clone showing highest binding affinity was selected and subjected for overexpression in *E. coli*. Simultaneously, this clone, A5, was sequenced and used to produce CDR3 by solid phage peptide synthesis. CDR3 is a small molecule that itself is difficult to produce in an expression system, so it was synthesized chemically. The absorbance (A_{450nm}) for NB in ELISA when allowed to interact with *Borrelia* was 3.8, while CDR3 had 3.7. NB could show low immunogenicity as their sequence is nearly identical to the human variable regions of the IgG heavy chain, however they often low level immune response could be observed. Use of only CDR3 as an antigen binding molecule may significantly reduce immunogenicity. A5_{CDR3} will be conjugated with a dendrimer and doxycycline will be encapsulated in dendrimer based nanosystem, to achieve targeted drug delivery against *Borrelia*. The A5_{CDR3} serve as molecules targeting *Borrelia*, while doxycycline has an effect for the treatment of Lyme borreliosis.

Innovative aspects

- Development of molecules targeting *Borrelia*, suitable for use in therapy.
- Development of a nanosystem for borreliosis therapy.

Qualitative and quantitative proteomic analysis of the *Escherichia coli* proteome after TFA cell lysis by LC-MS/MS

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Introduction

Bottom-up proteomics allows the study of all proteins that can be detected in cells and tissues. There are many studies in the literature on both prokaryotes and eukaryotes, however, most of the methods have been developed for the examination of eukaryotic cells. It would nevertheless be important to use and optimize these methods for analyzing bacterial cells. As prokaryotic cells differ from eukaryotes in many properties, studying their proteome poses different challenges. Many of them are human pathogens, so lysis buffers must completely inactivate both vegetative cells and spores. Studying bacteria alone and their interactions with various other microorganisms can provide new insights into their impact on human health. In this study, we examined *Escherichia coli* bacteria forming three different phenotypic colonies at the whole proteome level.

Methods

All *Escherichia coli* samples were lysed in 100% trifluoroacetic acid (TFA). The total protein content of the samples was determined using BCA Protein Assay Kit followed by an in-solution digestion. Tris(2-carboxyethyl)phosphine (TCEP) and chloroacetamide were used for protein denaturation and alkylation. All measurements were carried out on a Waters ACQUITY UPLC M-Class LC system coupled with an Orbitrap Exporis™ 240 (Thermo Fisher Scientific) mass spectrometer. Peptides were separated on an 85-min nanoflow liquid chromatography gradient and analyzed in data-dependent (DDA) and data-independent (DIA) acquisition modes. Protein quantification was performed with DIA-NN using a predicted spectral library, while the effects of unexpected peptide modifications and nonspecific cleavages were estimated based on open searches of DDA data using Fragpipe.

Results and Discussion

About 50% of all *Escherichia coli* proteins were quantified with at least two unique peptides in all samples with a low coefficient of variation. Further comparisons were made on these proteins. In addition to comparing the proteome of different *Escherichia coli* phenotypes, the unexpected modifications and non-specific cleavages due to TFA homogenization, which can have a significant impact on the quantitative results, were investigated. The high reproducibility of sample processing protocol and peptide selection based on statistical parameters enabled the identification of a large number of proteins with significantly different expression levels in the studied states of *Escherichia coli*.

Innovative aspects

- *Escherichia coli* cells lysed with TFA were digested in solution and their total proteome was analyzed by nanoLC-MS/MS

Comparative Transcriptomic and Proteomic Analyses of *Saccharomonospora azurea* strains in terms of primycin producing ability

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Introduction

Due to the rise of antimicrobial resistance, there is an urgent need to discover new agents for combating bacterial infections. Actinomycetes, known for their ability to synthesise over 10,000 bioactive agents, have a pivotal role in this pursuit. Among them, *Saccharomonospora azurea* is known as an exclusive producer of primycin a 36-membered non-polyene marginolactone antibiotic with high antimicrobial activity against frequent Gram-positive pathogens, including clinically prevalent multidrug-resistant strains. Although our previous research highlighted the structural and functional genomic aspects of enhanced primycin production by *S. azurea* SZMC 14600 strain, to unravel in depth the regulation of primycin synthesis, we conducted a comparative study integrating transcriptomics and proteomics data. Transcriptomic analysis revealed potential protein-coding genes, including those associated with secondary metabolism. Proteomic analysis during primycin fermentation provided insights into differentially regulated proteins. This multi-omics approach enhances our understanding of antibiotic production and microbial responses, offering new avenues for therapeutic development.

Methods

Bacterial strains and culture conditions Culture conditions for the primycin producer *Saccharomonospora azurea* SZMC 14600 and *Saccharomonospora azurea* DSM 44631 strains were carried out according to Valasek et al.

Transcriptomic analysis Total RNA was extracted from five days old fermentation extracts. High-quality total RNA samples (RIN >8.5) from pooled biological replicates were processed using the SOLiD total RNA-Seq Kit.

Bioinformatic analysis RNA-Seq data was analyzed using Galaxy's open-source, web-based platform. Transcriptome assembly and differential expression analysis were performed according to the Cufflinks RNA-Seq workflow.

2D Gel Electrophoresis Isoelectric focusing of 250 µg of whole protein was performed by Protean IEF system. Differential protein spots were excised from the gel and in-gel tryptic digestion was performed. Peptide mass fingerprinting by MALDI-TOF/TOF MS Proteins from MS/MS spectra were identified by ProteinScape 2.1 server utilizing the MASCOT PMF database search software and Bruker BioTools 3.2 software accessing the MSDB, Swiss-Prot and NCBI nr databases.

Results and Discussion

To gain more insight into gene expression differences between high and low primycin producer *S. azurea* strains, RNA Seq has been applied. Transcriptomic analysis revealed 330 and 356 differentially expressed genes in a set of *S. azurea* SZMC 14600 vs. *S. azurea* DSM 44631 respectively, classified into diverse GO and COG categories. To further support the results obtained at the transcriptional level 2D gel electrophoresis followed by peptide mass fingerprinting was performed. Accordingly, 5-5 spots were selected and analysed in the case of each *S. azurea* strain. The results were in good agreement with transcriptomic analysis in the case of 7 protein spots out of 10, while in the case of 3 proteins the observed expression pattern could not be elucidated by transcriptomic profile.

Innovative aspects

- Integrated transcriptomics and proteomics data provide deeper insights into the primycin biosynthetic process.

Paramagnetic beads in proteomic sample preparation

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Introduction

The main difficulties of performing proteomic analyses on biological samples are their low availability and the complex sample preparation process required to make them suitable for mass spectrometry (MS) analysis, steps that often result in significant losses, which further reduces the amount of material that can be used for measurements. To overcome these problems, it is essential to develop new, state-of-the-art sample preparation methods with reduced losses.

Methods

In the literature, some methods can be found that meet the above requirements, such as sample preparation protocols based on the use of paramagnetic beads, abbreviated SP3¹ and SP2². Although these methods are considered promising candidates for the treatment of biological samples, their major shortcoming is that they have not been proven to be universally applicable for the preparation of different biological samples. While the SP3 method is suitable for the purification and enzymatic digestion of protein samples, its applicability for the clean-up of peptide samples is unclear. The SP2 method is recommended in the literature for the purification of peptides but has not been used previously for the purification of protein samples. Furthermore, there is no comparative study in the literature evaluating these sample preparation methods for biological samples with different hydrophilicity.

Results and Discussion

The aim of our research was to compare the methods described above and to develop a novel proteomic sample preparation and purification method based on paramagnetic beads, which can be used for the preparation of both protein and peptide samples of different origins for subsequent MS measurements. We have shown that using either SP3 or SP2 methods, we can identify a higher number of proteins than with the commonly used in-solution digestion and C₁₈ purification protocol, if the sample preparation is performed on proteins for SP3 and on peptides for SP2. If this is changed, that is no longer the case. However, the performance of the different methods strongly depends on the type of the biological sample analyzed, as not the same methods resulted in the highest protein hits for liver cell lysate and extracellular vesicle samples. Finally, we have developed a paramagnetic bead-based protocol, which can be applied for the preparation of both protein and peptide samples. The established sample preparation method differs from and outperforms previous methods in the literature.

Innovative aspects

- A detailed comparison between protocols using paramagnetic beads that can be found in the literature.
- Development of a sample preparation protocol that can be applied to both protein and peptide samples.

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Optimisation of an AP-XL-LC-MS/MS workflow to study the architecture of native LAMTOR assemblies

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Introduction

The late endosomal and lysosomal adaptor and MAPK and mTOR activator (LAMTOR) scaffolding complex, resides at the lysosomes where it mediates catabolic and anabolic signalling. The pentameric complex consists of LAMTOR1 whose lipid-modified N-terminal region anchors the complex together with the remaining subunits LAMTOR2-5 in the lysosomal membrane. Previous studies have identified the presence of at least four different LAMTOR assemblies on the lysosomes associated with catabolic or anabolic signalling by associating with Rag-GTPases, SLC38A9, the v-ATPase, AXIN and LKB1 as well as lysosomal biogenesis and cellular positioning via BORC. Defects in pathways associated with these assemblies have been linked to multiple pathological phenotypes including neurodegeneration, cancer, infection, immunodeficiency, and obesity. Thus, a better understanding of physiological lysosomal signalling might reveal new therapeutic strategies.

Methods

We aim to investigate the architecture of native LAMTOR assemblies upon different stimuli to understand how a single scaffolding complex can regulate multiple biological functions. Ultimately, this will help us in understanding how patient mutations might affect these tightly regulated interactions. We plan to capture these endogenous PPIs by employing state-of-the-art crosslinking mass-spectrometry (XL-MS/MS) methods of affinity purified protein complexes or FeDEX isolated lysosomes. Data analysis is performed using XlinkX, Annika 2.0 and MeroX to assess the comparability as well as the reliability of different algorithms.

Results and Discussion

We generated hTERT-RPE1 cell lines endogenously expressing ALFA-tagged LAMTOR and BORC subunits. We are able to purify ALFA-tagged proteins and their binding partners in a highly selective manner using anti-ALFA magnetic beads. My current focus is to embed the purification within an elaborate LC-MS compatible workflow including crosslinking and digestion on ALFA-magnetic beads. Furthermore, LC-MS/MS conditions including FAIMS isolation of charged species and HCD fragmentation for identification of MS-cleavable crosslinked peptides are being tested.

Innovative aspects

- Endogenous Knock-In of ALFA-tagged LAMTOR components in hTERT RPE-1 cells via CRISPR-Cas9
- Affinity purification using ALFA-tag specific magnetic beads combined with on beads cross-linking and digestion
- Comparing the performance of different data analysis software for affinity purified crosslinked protein complexes

Characterizing HDAC1-driven proteomes, acetylomes, and phosphorylomes in CD4+ Th cells

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Introduction

To understand how HDACs affect T-cell-mediated immunity and control T-cells by regulating histones and non-histone proteins, we implemented TMT multiplexing (16-plex) protocols and harmonized them with downstream protocols for PTM enrichment, to enable simultaneous quantitative profiling of the proteome, acetylome, and phosphorylome in CD4+ T-cell lineages (naïve, and in vitro differentiated Th1, Th2, Th17, and Treg) in wildtype and HDAC1 knockout backgrounds.

Methods

To optimize the quantitative accuracy of this method, we implemented a combination of FAIMS with Real-Time Search (RTS) for analysis of the proteome fractions, achieving high quantitative accuracy. However, for PTMs, a method using FAIMS and MS2 was found to perform much better. To address the issues of ion interference inherent in MS2-based measurements of isobaric tagging approaches, we implemented an in-house computational model pipeline that allows quantification and correction of interference for any given multiplex dataset.

Results and Discussion

Ultimately, we generated a CD4+ T-cell subset-specific atlas of the acetylproteome, phosphoproteome, and proteome. We were able to quantify roughly 8000 proteins and up to 11500 acetylation and 15000 phosphorylation sites across all experimental conditions. The data revealed lineage-specific proteome, acetylome, and phosphorylome profiles. Additionally, we determined the impact of HDAC1 deletion on these signatures.

Innovative aspects

- CD4+ T-cell subset-specific atlas of the acetylome, phosphorylome, and proteome.
- Th lineage -specific profiles.
- Impact of HDAC1 on Th lineage specific signatures.

Proteome signatures of epicardial adipose tissue (EpAT) across six different heart-regions from patients undergoing heart transplantation

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Introduction

Coronary artery disease (CAD) has become the leading cause of morbidity and mortality worldwide. Over- and malnutrition in combination with reduced physical exercise promote metabolic imbalances in favor of the accumulation of adipose tissue (AT). As AT acts as an endocrine organ in a tissue-specific manner, we aimed to profile the protein repertoire of the epicardial fat (EpAT) to obtain molecular signatures characterizing six different topologies of the failing heart. As AT is a causal factor in the development and progression of atherosclerosis including CAD, region-specific protein compositions could help to understand not only the pathologic, but also the nourishing factors of EpAT.

Methods

Proteaneous material from adipose tissue collected from Aorta Ascendens, Apex, Coronary Sinus Vein, Left Ventricle and tissue in vicinity to a previously implanted mechanical support (LAD) as well as Right Ventricle from three patients undergoing heart transplantation was sequentially processed by delipidation (MeOH) and lysis (SDC) in Precellys and subjected to in-gel as well as in-solution/SP3 tryptic digestions followed by high resolution mass-spectrometry analysis in data-dependent and -independent analysis on Tims ToF Pro and Orbitrap Exploris instruments.

Results and Discussion

We found a hitherto unrecognized protein complexity in EpAT from the six cardiac regions and identified more than 6000 different proteins. We observed an EpAT-region and patient-specific proteome. These results will assist efforts to exploit EpAT for in-vitro three-dimensional adipo- and cardiac organoids culture screening platforms to uncover new pharmaceutical treatment options.

Innovative aspects

- Combining traditional in-gel with popular in-solution/SP3-digestion facilitates the identification of charged, hydrophobic, nucleic acid bound proteins often underrepresented in tissue proteomes. Thus, it is worth to invest hands-on-time in sample preparation.
- Dissection and comparative analysis of regional EpAT depots provides the basis to understand spatially specialized nourishing functions of EpAT.
- Although a high interpatient-variability impedes general conclusions, pilot studies provide a valuable resource for design and stratification when planning larger clinical studies.

Proteomics Assisted LNP Screening (PALS)

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Introduction

LNPs have been demonstrated to hold great promise for the clinical advancement of RNA therapeutics. Continued exploration of LNPs for application in new disease areas requires identification and optimisation of leads in a high throughput way. Currently available high throughput in vivo screening platforms are well suited to screen for cellular uptake but less so for functional cargo delivery. We report on a platform which measures functional delivery of LNPs using unique peptide 'barcodes'. We describe the design and selection of the peptide barcodes and the evaluation of these for the screening of LNPs. We show that proteomic analysis of peptide barcodes correlates with quantification and efficacy of barcoded reporter proteins both in vitro and in vivo and, that the ranking of selected LNPs using peptide barcodes in a pool correlates with ranking using alternative methods in groups of animals treated with individual LNPs. We show that this system is sensitive, selective, and capable of reducing the size of an in vivo study by screening up to 10 unique formulations in a single pool, thus accelerating the discovery of new technologies for mRNA delivery.

Methods

The sequence optimised mRNA encoding each barcoded protein- barcoded hEPO and barcoded CRE – was synthesized by in vitro transcription. Lipid Nanoparticles (LNPs) were prepared using a microfluidic chip device, NanoAssemblr. For the in vitro transfection of HEP G2 cells and HEK 293 cells, lipofectamine complexed mRNA and LNP containing the desired mRNA were used. Purified barcoded hEPO was generated by transient transfection of plasmids encoding human EPO with C-terminal barcodes followed by a FLAG tag in a HEK293 derived cell line (Patent: US 2020/0370056 A1). Human EPO levels in cell culture medium and from mouse serum samples was analysed using ELISA. 200µl of serum per mouse was processed using an EPO enrichment column. Purified EPO protein was quantified by ELISA and trypsin digested and subjected to mass spec analysis. Peptides of interest were manually searched in Total Ion chromatogram (TIC) and precursor ions was extracted for each peptide of interest. Peaks were manually integrated to obtain the peak area and intensity.

Results and Discussion

we have presented a design for peptide barcoding which enables a more efficient screen of LNPs and potentially other nucleic acid delivery systems. We show that the barcoded mRNA is efficiently translated into barcoded proteins which remain functional, and the relative amount of each peptide barcode accurately represents the relative efficacy of the associated LNP. Indeed, the apparent differences in rate of translation did not affect the ranking of LNPs in a pool. We demonstrated that we could administer up to 10 LNPs in a pooled dose to mice and obtain the same outcome as if we administered the same 10 LNPs individually, despite the variations due to mRNA translation and the relative dose-dependency of LNP efficacy. Furthermore, we show that PALS does not induce toxicity and in fact, is a safe way to screen for the efficacy of multiple LNPs at a fraction of the dose, cost, time and lab animals used.

Innovative aspects

- PALS was designed to assess the hypothesis that a pool of LNPs administered to test animals can reliably determine the functionality of individual LNPs with a similar outcome as when the LNPs are administered individually to groups of animals. To test this hypothesis, we answer three key questions:
- Will barcoded mRNA be efficiently translated to functional barcoded proteins?
- Can the barcode measurement by mass spectrometry be used as a surrogate for protein quantification?
 - Is PALS sensitive enough to quantify and differentiate LNPs in a pool?

Effects of CKD-derived uremic toxins on endothelial cells and THP-1-derived M0 macrophages

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Chronic Kidney Disease (CKD) is defined as the progressive loss of renal function persisting for more than 3 months, ultimately leading to kidney failure and the need for renal replacement therapy. However, the main cause of mortality in CKD patients is cardiovascular disease-related complications. Accumulation of uremic toxins in CKD accelerates endothelial dysfunction, driving the atherogenic processes and leading to cardiovascular events, like stroke or myocardial infarction. Systemic inflammation and infiltration of lymphocytes in the sub-endothelial space enhances endothelial dysfunction and atherogenesis. We have previously demonstrated that the dynamics of cell transmigration through endothelium differ in CKD and classical cardiovascular disease (CVD) (Tracz et al. 2021). Here, we have investigated the effects of CKD-patient-derived serum on endothelial cells (HUVEC) and THP-1-derived M0 macrophages. We have observed a decrease in HUVEC viability upon CKD and CVD sera treatment. CKD serum treatment also caused an increased inflammasome response in HUVEC. The cell cycle pattern of HUVEC was found to be dysregulated. Upon proteomic analysis, dysregulation of proteins involved in ribosome-related pathways, lipid metabolism, RNA metabolism and cell cycle were revealed as altered between HUVEC cells treated with serum from CKD and CVD patients. Finally, a transwell cell invasion study on the xCELLigence system with THP-1 derived M0 macrophage showed a significant difference in cell migration induced by CKD, CVD, and control sera. The obtained results support the hypothesis that distinct mechanisms underlie the acceleration of CKD-related and non-CKD-related atherosclerosis.

Combining Bioenergetic Measurements with Untargeted Metabolomics for Illuminating Cell Response to Drug Treatment

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Introduction

To better understand the biology of cancer cells and their dynamic metabolic response to therapeutic compounds, advanced methodologies are required. We combined results from two technologies, Seahorse XF Pro cell analyzer and Revident LC/Q-TOF, that measured metabolic pathway utilization at two scales, cellular and molecular.

Methods

An acute monocytic leukemia cell line was treated with the drugs SU1498 and AG-879, which were selected from a library of 80 kinase inhibitors based on their modulation of ATP production rates. The converted data provide a quantitative overview of drug treatment impact on cellular-level energetic phenotypes.

Separately, cells treated with the drugs were lysed, metabolism was quenched, and lipids and metabolites were extracted with an automated sample preparation method on the Bravo platform. Extracts are then separated with an 1290 Infinity II Bio LC system and directly analyzed with a novel combination of the new Revident LC/Q-TOF and MassHunter Explorer software.

Results and Discussion

The combined results provide deeper insight into the cellular and molecular metabolic response to drug treatment. Seahorse XF analysis of AG-879 and SU-1498 corroborated previous results and newly demonstrated that both drugs cause mitochondrial uncoupling. Untargeted Metabolomics identified changes in key metabolites affected in glycolysis and mitochondrial respiration that correlate with XF results. Untargeted Lipidomics showed an increase in TG content with SU-1498 treatment which may be related to buildup of energy precursors for the TCA cycle, shown to be reduced with XF.

Proteomic and metabolomic status of BY2 cells adapted to long-term NaCl stress

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Introduction

In the coming decades, water scarcity and osmotic stress are expected to increasingly impair plant cell functions. Despite extensive research on plant cell responses to water stress, our understanding of the molecular adaptations enabling cells to endure chronic adverse conditions remains limited.

In this study, we examine tobacco BY2 suspension cells adapted to osmotic stress since 2006. These cell lines serve as an ideal model to explore the molecular mechanisms of osmotic stress adaptation. It is believed that adapted plants/plant cells achieve the status of "new molecular homeostasis". However, the molecular status of adapted plant cells and the reflection of the postulated state of new homeostasis on the proteome and metabolome of the years-adapted cells remain largely unknown.

Methods

We utilized an untargeted GC/MS analysis to study BY2 line years- adapted to 190 mM NaCl and controls. The high-throughput metabolome study was supplemented with biochemical study of selected stress markers, radical levels, etc.

Proteomic study focus on high-throughput gel-free comparative analysis, but were complemented by western blot analysis to analyze the amount of nitrotyrosines and the amount of selected antioxidant enzymes.

Results and Discussion

The abundance of numerous core metabolites was unchanged in adapted cells compared to controls suggesting the "stable state" of essential cellular molecular pathways. Nevertheless, the clear signals in cells' adaptation to osmotic stress were visible with significantly up-regulated proline, sorbitol or β sitosterol. Cells exposed to years-long stress show higher MDA levels without increased radicals, indicating most probably unknown defense mechanisms distinct from those in short-term stress responses.

In the same time proteome of adapted cells was even more similar to controls than metabolome, with only a few proteins differed in their abundance in adapted BY2 cells. We did not detect alerted level of analyzed 'protein-related' stress marker: nitrotyrosine or abundance of selected anti-oxidative enzymes.

The stress-adapted BY2 cells are significantly smaller than cells under normal conditions, resembling many plants under chronic stress. It's believed that the reduction in cell size and mass is the "cost" of responding to stress. However, the energy-related proteins/metabolites levels were generally unchanged in adapted to NaCl BY2 line. Thus the source of energy for the biosynthesis of stress-related compounds remained still not fully recognized in adapted cell line.

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Innovative aspects

- Increased levels of compounds enabled cells to function in higher osmoticum were found in adapted cells.
- The metabolome and proteome changes reflect a new molecular homeostasis, as the core metabolic pathways remain largely unchanged at the OMIC level.

Development of functional CDR3-derived proteins against SARS-CoV-2

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Introduction

Drug delivery across the blood-brain barrier (BBB) continues to be difficult, making neuroinfection treatment challenging. Conventional antibodies are ineffective in this setting due to their large size (150 kDa), which limits their biodistribution in the central nervous system (CNS). Nanobodies derived from the variable domain of heavy chain only antibodies found in Camelidae offer a promising alternative in the treatment of neuroinfections thanks to their small size (15 kDa). Above all, their complementarity-determining region 3 (CDR3; ~5 kDa) plays a dominant role in binding nanobodies to antigens. The goal is to produce CDR3 capable of binding the SARS-CoV-2, fuse it with the Fc domain of human IgG1 to increase stability, and angiopep-2 to enhance translocation of the fusion protein from blood to the CNS across the BBB.

Methods

RNA was extracted from peripheral blood mononuclear cells (PBMC) of *Llama glama* that had been immunised with SARS-CoV-2 protein. To create a CDR3-*E. coli* library, cDNA was reverse transcribed from RNA, the fragment coding CDR3 was amplified with degenerated primers, digested with *NcoI* and *NotI* restriction endonucleases, cloned into pSEX81 phagemid containing Fc fragment, and transformed into *E. coli* XL1-blue. Library was superinfected with M13 K07ΔpIII Hyper Phage to escape the packaged phage particles displaying CDR3 on their p3 minor coat protein. To select the specific CDR3-phages against SARS-CoV-2, two rounds of panning against inactivated virus were performed. Interaction of phages to SARS-CoV-2 was confirmed by phage ELISA. DNA from phages after last round was isolated and CDR3 in frame with Fc domain and angiopep-2 was amplified. Amplicon was ligated into an expression cassette and used to transform *Pichia pastoris* to overexpress protein in secreted form. A total of 96 CDR3-Fc-angiopep-2 clones were screened for their ability to bind protein S and shortlisted clones were tested for their ability to neutralize pseudovirus.

Results and Discussion

Using degenerated primers a 120 bp product corresponding to size of CDR3 was amplified. Product was used to generate a CDR3-*E. coli* library. The size of the library was 1.2×10^{10} , which was used to construct a CDR3-phage library. After the 2nd round of panning virus specific CDR3-phages were enriched, which was assessed by phage ELISA (Absorbance^{450nm} 2.37 against 0.4 of negative control). The CDR3 in frame with Fc domain and angiopep-2 amplified from enriched phages had 1080 bp. The CDR3-Fc domain-angiopep-2 secreted from *Pichia pastoris* had approximately 32 kDa. Of the 96 clones tested for binding ability to protein S, 15 showed significant affinity. Four of the shortlisted clones showed virus neutralisation of at least 80%. These four clones are being studied for their EC₅₀ against live virus, cell toxicity, and ability to cross the BBB in vitro. Research was funded from APVV-22-0084, VEGA1/0381/23, VEGA1/0348/22, EURONANOMED2021-105.

Innovative aspects

- Enhancing delivery of antiviral molecule into the brain by fusing it with Fc and BBB homing peptide angiopep-2.

Investigation of lipid mediators for Crohn's disease

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Introduction

The accelerating prevalence of Crohn's disease (CD) in newly industrialised countries in the Middle East, South America and Asia ¹ has contributed to an enhanced urgency to understand the pathophysiology of inflammatory bowel diseases (IBDs). Crohn's disease, together with Ulcerative Colitis, make up the most common types of IBDs. The number of researches, focusing on these types of diseases, especially on Crohn's disease is limited and a broader overview is needed for a better understanding of the disease. To unravel potential biochemical key mechanisms of the disease and to have a detailed look into them, understanding the interplay of various analytes is likely to be crucial. Therefore, in this study we applied a combined untargeted proteomics and oxylipin approach in blood serum and tissue samples of patients with Crohn's disease.

Methods

Untargeted proteomics and lipid mediator analysis was applied of serum and tissue samples, using LC-MS-based approaches. Tissue samples were collected from actively inflamed areas and normal-appearing mucosa regions from 13 CD patients. Blood serum samples were collected at the date of surgery and subsequent endoscopy, approximately 5 months after surgery.

Results and Discussion

Serum proteomics showed no evidence of any significantly altered proteins between the surgery and endoscopy date. In comparison, the oxylipin analysis of the serum revealed regulatory events with significantly upregulated arachidonic acid (AA)-derived oxylipins, including cyclooxygenase (COX)- and 5-lipoxygenase (LOX) products. The 5-LOX pathway is described to be specifically involved in inflammatory processes, whereas the COX-derived products can also be formed independent of inflammation ^{2,3}. This oxylipins signature demonstrates the tendency of Crohn's disease patients for progredient inflammation.

Innovative aspects

- The combined analysis of proteins and oxylipins supported the identification of novel
- blood-borne biomarkers suitable for disease monitoring

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Optimization of SPME technique towards efficient quantification of phytocannabinoids in growing medicinal cannabis plants

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Introduction

Solid Phase Microextraction (SPME) is an interesting sample preparation approach for the monitoring of concentrations of phytocannabinoids (PCs) in complex biological matrices [1]. SPME involves extraction of analytes into thin, biocompatible probes coated with sorbent (extraction phase), and is suitable for the *in vivo* experiments. In SPME, quantification of analytes is based on the equilibrium between the sample matrix and the coating. This enables efficient quantification of analytes even when the sample volume is unknown. However, to obtain reliable results, the SPME protocols require insightful optimization. In this study, the impact of the coating SPME probes and the sample volume for efficient isolation of four PCs (tetrahydrocannabinolic acid, cannabidiolic acid, cannabigerolic acid, tetrahydrocannabivarinic acid) from plant material was examined. In addition, extraction time profiling (ETP) was performed on the living cannabis plants to select the optimum time for the extraction of PCs.

Methods

In-lab made SPME probes were prepared according to the internal protocol. The extraction time profiling was conducted on growing THC-dominant cannabis plants cultivated under control conditions in the Cannabis Facility, International Clinical Research Centre, Brno, Czech Republic. The C18 and HLB coated SPME probes were inserted into living plants for 10, 20 and 30 minutes of static extraction of four PCs. The influence of sample volume was examined on homogenised and non-homogenised cannabis plant material. For that purpose, SPME probes were initially placed into non-homogenised cannabis inflorescences for 20 min static extraction. Next, inflorescences were homogenized, and portions of 1, 2 and 4 grams of homogenised cannabis plant material were subjected to SPME sampling. Desorption of the analytes and final instrumental analysis of extracts were conducted according to previously optimized methodology [1].

Results and Discussion

ETP experiments performed on growing cannabis plants revealed that 10 min extraction with the use of C18 probes provides the best results in terms of extraction efficiency of PCs. HLB coating provided slightly better extraction efficiency, but higher RSD values for studied analytes were observed. Therefore, for further experiments C18 coating was chosen. The analysis of the impact of sample volume on the extraction efficiency of PCs from homogenized plant material showed that there is no dependence in term of the extraction efficiency of sampling performed from 1, 2 or 4 grams of plant homogenate. This is in agreement with previous observations for other complex matrices that SPME is an excellent tool for quantification of analytes in living system.

Innovative aspects

- It has been demonstrated that SPME technique is an excellent tool for extraction of PCs *in vivo* conditions with the use of in-lab made C18 probes, and its efficiency is independent on the volume of the sample matrix.

- References:

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Unlocking the proteomic potential of FFPE tissues with BeatBox® and iST: A xylene-free, high-throughput workflow for in-depth proteome analysis

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Introduction

Access to formalin-fixed, paraffin-embedded (FFPE) tissue materials is relatively easy, as it is evaluated, preserved, and stored according to well-established procedures in routine clinical diagnostics. FFPE tissue sample preparation for proteomic analysis, however, is extremely challenging and harsh conditions are required to remove cross-links and paraffin for efficient protein extraction. By combining the BeatBox tissue homogenizer with an optimized iST workflow for proteomic sample preparation, it is now possible to process 96 samples of FFPE-tissue per working day easily, efficiently, and reproducibly without using toxic xylene-based deparaffinization. In combination with state-of-the-art LC-MS instrumentation, acquisition methods, and data-processing pipelines, biomarker discovery from FFPE-tissues reaches a completely new level in the field of proteomics.

Methods

FFPE samples (10 µm curls, non-deparaffinized) from mouse tissues (cardiac muscle, kidney, and liver) and matching snap-frozen tissue samples (1-2 mg pieces) were processed in 96-well format using BeatBox homogenization coupled to iST sample preparation. For FFPE samples, an optimized workflow was established: FFPE curls were homogenized in the BeatBox (high power, 10 minutes), followed by an one-hour incubation at 80-95 °C, to de-crosslink, extract, reduce and alkylate proteins. Applying the iST sample preparation protocol, tryptic digestion was followed by an optimized peptide clean-up with an additional washing step to remove last traces of paraffin. Peptides were analyzed on a nano-LC coupled to a timsTOF mass spectrometer using diaPASEF® acquisition mode. Raw files were analyzed using Bruker ProteoScape™.

Results and Discussion

To evaluate the performance of this novel xylene-free FFPE workflow using BeatBox and iST, it was compared to a traditional workflow with xylene-based deparaffinization and bead-based sonication. For all tested tissue types, BeatBox homogenization outperformed bead-based sonication, revealing an increase in protein IDs of >10%, with a remarkable increase of 43% for mouse cardiac muscle tissue. Notably, the xylene-free BeatBox-iST protocol achieved excellent intra- and interday repeatability with median CVs of <10% (n=4). Besides, it could be ensured that paraffin was completely removed from FFPE samples and no contamination was introduced into the LC-MS instrumentation.

Comparing FFPE tissues with the corresponding fresh-frozen tissues revealed more than 10'000 proteins for both conditions (fresh-frozen vs. FFPE) with up to 73% common proteins and a similar dynamic range, confirming the superior performance of the FFPE BeatBox-iST workflow.

Innovative aspects

- This BeatBox-iST workflow simplifies large-scale retrospective proteomic studies by providing a xylene-free, robust and high-throughput solution for FFPE tissue samples.

Unique high-throughput workflow for deeper plasma/serum proteome coverage enables discovery of novel biomarkers

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Introduction

Blood plasma represents an invaluable source for proteomic biomarker discovery as it is easily accessible and provides comprehensive information about an individual's health status. However, the high dynamic range as well as sample heterogeneity and complexity pose significant challenges for LC-MS-based proteomics, limiting access to the entire proteome. Moreover, common plasma workflows require tedious manual processing and are often incompatible with automation or the analysis of large cohorts. Here, we present the ENRICH-iST workflow as a robust solution to the dynamic range challenge in plasma samples based on enriching low-abundance proteins. This workflow enables high-throughput sample preparation for LC-MS-based proteomics with improved coverage of the plasma proteome, making it ideal for large cohorts in clinical research studies.

Methods

Starting from 10-20 μ L of plasma, the ENRICH technology allows efficient dynamic range compression by enriching low abundance proteins onto non-functionalized paramagnetic beads. Upon enrichment, proteins were further processed according to the iST-BCT protocol optimized for biofluids (PreOmics). Similarly, 2 μ L of neat plasma was also processed according to the iST-BCT protocol as a comparison. Peptides were analyzed by a nanoLC coupled to a tims TOF HT instrument using diaPASEF® acquisition mode. Data processing was performed with Spectronaut® 17 (Biognosys).

Results and Discussion

The novel ENRICH technology offers a high-throughput, robust and reproducible method to enrich low abundance proteins in plasma for improved biomarker discovery. The performance of the ENRICH -iST kit compared to neat plasma (iST-BCT) was assessed in a clinical cohort of human plasma from lung cancer patients (n=10) and age-matched healthy donors (n=10). Approximately 1470 protein groups were identified in ENRICH plasma compared to ~660 protein groups in neat plasma which reflects an improvement in identification of 2.2-fold. Proteome depth could be further increased with ~1980 protein groups identified or a 2.9 fold increase compared to neat plasma by utilizing a cohort-specific spectral library for data processing. Library-based processing also improved data quality with 60% of all protein groups identified across $\geq 80\%$ of samples containing ≥ 2 peptides/protein. Increased proteome depth is achieved by the enrichment of low abundance proteins on paramagnetic beads which simultaneously reduces the concentration of high abundance proteins. The contribution of the top 20 proteins with the highest intensities in neat plasma to the total protein intensity dropped by roughly 20 percentage points from ~56% to ~37% in ENRICH plasma samples. The effective reduction in high abundance proteins by ENRICH facilitates the identification of otherwise hidden low abundance proteins such as cytokines with an additional 13 cytokines being detected in ENRICH plasma. Statistical analysis of the quantified proteins for the ENRICH-iST dataset showed a clear stratification between healthy donors and patients, and compared to the neat dataset, 16 additional significantly regulated proteins were identified as potential biomarkers. Strongly upregulated factors in the lung cancer samples included S100A8 and S100A9, which have previously been reported to be upregulated in lung cancer and other types of cancer (<https://doi.org/10.1016/j.bcp.2006.05.017>).

Innovative aspects

- By combining high-throughput with deep proteome coverage, the ENRICH-iST workflow for plasma/serum proteomics boosts biomarker discovery in (pre)clinical studies.

Simultaneously looking at molecular signature of HIV during long-term immune monitoring of patients undergoing antiretroviral therapy (ART)

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Introduction

Regular monitoring of HIV patients receiving continuous antiretroviral therapy (cART) is prerequisite to ensuring patient benefits and long-term effectiveness of ART treatment. Interrogation of such patient samples for molecular signature of the disease together with clinical parameters such as mitochondrial mass, incomplete immune reconstitution, and CD4+ T-cell counts in people living with HIV (PLWH), may help better understand disease progression and efficacy of treatment. The ability to simultaneously measure corresponding gene expression levels of HIV together with disease-impacting proteins from patients' blood can add tremendous value to comprehensive analysis of disease progression.

Methods

Here, we demonstrate messenger RNA (mRNA) expression using QuantiGene™ assay on a multiplexing platform. Human peripheral blood mononuclear cells (hPBMCs) stimulated with phytohemagglutinin (PHA) were harvested at 24 hours and assayed for specific mRNA targets on a QuantiGene Human 8plex panel. Quantification of CCL2, CLDN5, CSF2, CXCL8, IL17F, IL25, IL6 and OCLN genes were performed on a Luminex 200 system.

Additionally, whole blood samples from PLWH on continuous ART (A, n=15), with CD4AC >500 cells/μl and CD4/CD8 ratio > 0.9, or untreated (B, n=10) were collected during routine immune monitoring. Mitochondrial mass (MM), mitochondrial membrane potential (MMP) and mitochondrial superoxide levels (MSL) in CD4 and CD8 T cells were determined using Mitotracker Green FM (M46750), Mitotracker Red (M22425) and MitoSOX Red (M36008), respectively using multicolor flow cytometry on FACSCanto II.

Results

To test the fidelity between Antiretroviral therapy (ART+ / ART-) and corresponding mRNA expression levels, we observed the CD4 absolute count and the CD4/CD8 ratios. Mean CD4AC and CD4/CD8 ratio in HIV+ART+ were within the reference ranges and differed significantly as compared to HIV+ART- (955 vs 388, $p < 0.0001$ and 1.478 vs 0.378, $p < 0.0001$, respectively). Untreated HIV infection was associated with significantly increased expression of CXCL8 mRNA (7.33 vs. 3.82, $p < 0.05$). Interestingly, HIV+ART+ individuals with CD4 AC < 1000 had a significantly higher expression of CSF2 and IL-6 as compared to treated patients with CD4AC >1000 (15.67 and 15.20 vs 7.72 and 8, $p < 0.05$ for both). In the whole blood study, no difference was found in MMP of CD4 and CD8 T lymphocytes. However, the mean fluorescence intensity (MFI) of MM of CD8 T lymphocytes differed significantly between cART+ and cART- (2308 vs 6396, $p < 0.05$). MFI of CD4 MSL, as well as the percent of CD4 and CD8 T lymphocytes with detectable MSL, were elevated in cART- compared to cART+ (1889 vs 1258, $p < 0.05$, 38.1 vs 11.3, $p < 0.001$, 20.9 vs 10.6, $p < 0.05$, respectively).

Discussion

Increased MSL in T lymphocytes of cART- PLWH suggested elevated oxidative stress and possible mitochondrial damage. Additionally, higher MM found in cART- suggested mitochondrial health disruption as well as on-going biogenesis. Complementing whole blood assays with hPBMCs based QuantiGene mRNA assay, provided a more holistic view of the antiretroviral therapy for HIV by helping to screen for molecular signature of the disease at gene level. Such a combined approach can elevate patient blood sample screening to a 'multi-omics' level, providing a high-level interrogation of clinical parameters concurrently with gene expression networks of the disease.

Innovative aspects

- HIV blood screening at 'multi-omics' level

The **BASP1** signaling protein interferes with the oncogenicity of **MYC**

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Introduction

MYC represents a transcription factor regulating crucial cellular processes like proliferation, differentiation, or apoptosis. While MYC activities in normal cells are essential and tightly regulated, MYC is frequently found to be deregulated in about 70 % of all human tumors, in which this oncoprotein represents a major cancer driver. One of the multiple transcriptional MYC targets is the gene encoding the brain acid-soluble protein 1 (BASP1) which is downregulated in a variety of MYC-dependent cancer cells. We found previously that ectopic BASP1 expression interferes with MYC-induced cell transformation.

Methods

Using the human colon cancer cell line SW480 featured by high MYC expression and a silenced *BASP1* gene, we further investigated the putative tumor-suppressive property of BASP1.

Three different cell types were established in which BASP1 is re-expressed. Whereas two cell lines are characterized by ectopic BASP1 expression, the third one was generated by CRISPR-mediated *BASP1* gene activation. Relevant cell lines were subjected to different biochemical methods including qPCR, RNA sequencing, immunoblotting and liquid chromatography coupled to mass spectrometry (LC-MS).

Results and Discussion

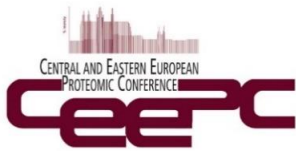
Expression of BASP1 in SW480 leads to a decrease of MYC protein and mRNA levels, and a significantly reduced transformed phenotype. Proteome comparison of SW480 cells with those ectopically expressing BASP1 (SW480_B) was performed using liquid chromatography coupled to mass spectrometry (LC-MS). From 4,543 analyzed proteins, 278 were found to be specifically activated in SW480_B including the tumor suppressor TP53. Among the 252 proteins downregulated in SW480_B are the MYC-associated factor X (MAX) and the metastasis-associated protein 1 (MTA1), the latter representing an oncogenic MYC target. Metabolome analyses from the same cell types revealed a potential effect of BASP1 on the glucose metabolism manifested by decreased lactate levels.

To obtain further insights into BASP1 function binding partners are analyzed by an immunological BASP1 protein pull-down followed by MS analysis.

Furthermore, we also test BASP1-mimetic peptides to develop strategies for the treatment of tumor cells featured by high MYC expression.

Innovative aspects

- Deciphering the mode of action of a potential tumor suppressor protein
- Uncovering novel interaction partners of BASP1
- Development of novel strategies for tumor treatment



CEEPC SCIENTIFIC COMMITTEE

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