Robust, Time-Efficient Approach for Protein Extraction from Fresh Human Skin Biopsies

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Comprehensive proteomic characterization of human skin is essential for understanding mechanisms of tissue homeostasis, repair, and disease, yet its clinical translation is limited by technically demanding and time-consuming protein extraction procedures. In this study, we developed a rapid extraction workflow and evaluated its performance against a conventional, multi-day RIPA buffer protocol. The optimized method, combining TPER-mediated lysis with enzymatic and mechanical dissociation, enables protein isolation from fresh skin biopsies in less than one hour. Data-independent acquisition mass spectrometry identified approximately 5,000 proteins using the rapid workflow, compared to ~6,300 proteins with the classical approach. A substantial overlap (68.6%) between datasets defined a conserved core skin proteome, indicating consistent recovery regardless of extraction chemistry. Both methods demonstrated strong reproducibility, supported by clustering and correlation analyses, while maintaining distinct quantitative profiles. Functional enrichment of shared proteins revealed key roles in cellular component biogenesis, metabolic processes, and RNA binding, with predominant localization to membrane-bound organelles and extracellular vesicles. Reactome pathway analysis further emphasized protein metabolism as a central feature. Importantly, the rapid workflow showed enhanced recovery of membrane-associated and protein complex components, which are relevant for biomarker discovery and therapeutic targeting. Although the classical protocol achieved greater proteome depth, its extended processing time limits practical application. In contrast, the rapid approach provides a scalable and clinically compatible alternative. Overall, this strategy preserves critical proteomic information while significantly reducing processing time, supporting its use in translational research and personalized approaches to wound management and skin disease.

NovoTax: prokaryotic strain identification from mass spectrometry-based proteomics data

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Traditional mass spectrometry-based proteomics relies on searching spectra against predefined protein databases. When sample composition is unknown, workflows typically depend on genome or 16S rRNA sequencing, or require using large databases that lead to lower peptide identifications due to the commonly used target-decoy approach. This limits the analysis of unknown isolates, mislabeled or contaminated samples, and complex communities. Although the accuracy of de novo peptide sequencing is improving, these approaches remain relatively niche, and translating predicted peptides into reliable taxonomic assignments and usable protein databases is still technically challenging.

To address this problem, we have developed NovoTax, an end-to-end Nextflow pipeline that identifies prokaryotic taxa directly from raw proteomics data and returns a sample-specific protein database for downstream searches. By combining de novo peptide sequencing with taxonomy-guided matching against large proteome databases, NovoTax recovers reported species and strain neighborhoods, identifies likely misannotations and contaminating organisms, and detects dominant members of microbial communities. By making these steps accessible in a single pipeline, NovoTax enables routine proteomics-based quality control and simplifies sample-specific database construction without requiring additional sequencing experiments.

Spatial and low input proteomics at scale with the timsUltra AIP

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Advances in ultra-high sensitivity MS, miniaturized sample preparation and fast chromatography enables proteome depth and sample throughput, crucial for large clinical studies. Here, we assess performance, scalability, and reproducibility of cellenONE-based sample preparation, separation the Evosep Eno coupled to a timsUltra AIP.

K562 digest dilutions, single FaDu and HeLa cells, and laser micro-dissected human FFPE tonsil tissue of T-cell, B-cell, and squamous cell epithelium were benchmarked. K562 digest was loaded on Evotips at 250pg to 50ng. FaDu and HeLa cells were isolated into single and 10 cell mini-bulks and human FFPE tonsil tissue (30,000 µm²) were prepared using cellenONE X1 Neo. Sample acquisition in dia-PASEF mode was performed on timsUltra AIP at different standard and Whisper Zoom gradients.

From the lowest K562 peptide load, 1,500 protein groups were identified at 500SPD, >4,500 at 30SPD, >4,000 at 120SPD, increasing to 5,500 at 40SPD with Whisper Zoom gradients. 50ng K562 identified 5,300 at 500SPD and >8,000 at 30SPD. In single FaDu and HeLa cells ~3,500 protein groups at 120SPD to ~5,100 at 40SPD were identified using spectra libraries.

Tissue contours from Human tonsil resulted in ~2,000 protein groups for the different cell type niches at 500SPD and increased to 6,000 at 30SPD. Biological differences between cell types were observable at any gradient length proving good quantitative performance at even the highest throughput. Among differentiating proteins, well-known biomarkers were identified (e.g. epithelium: Cadherin1, T-cells: CD3: B-cells: CD19), demonstrating excellent scalability and reproducibility of the cellenONE-Evosep Eno-timsUltra AIP workflow, enabling comprehensive large cohort investigations.

Exploring the proteomics capabilities of a new Trapped Ion Mobility Q-TOF designed for enhanced metabolomics performances

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The increasing use of multi-omics strategies in translational research drives demand for LC–MS platforms capable of high performance across varied analyte classes. Established timsTOF instruments excel in proteomics, while the newly developed timsMetabo focuses on improved transmission of low m/z analytes. The integration of the TIMS-MX cartridge, Athena Ion Processor (AIP), and Mobility Range Extension (MoRE) scan mode enables high-throughput analysis over an extensive m/z and 1/K0 range. This study investigates whether these advancements maintain robust proteomics performance, assessing timsMetabo's suitability as a versatile, high performance benchtop tool for multi-omics applications.

Samples were separated using a 25 cm x 75 µm column (Ion Opticks) on a nanoElute2 coupled to a timsMetabo, operating in dda-and dia-PASEF modes. Varying amounts of K562 digest were analyzed with a 22-minute gradient. For dia-PASEF, 24x25Th windows covering 400–1000 m/z were distributed across 8 ramps; dda-PASEF employed 10 ramps with 300-Hz acquisition. Data analysis was performed with DIA-NN 2.0 and FragPipe v22.0.

Injections ranging from 10 to 200 ng of K562 digest revealed a fivefold increase in signal intensity compared to previous timsTOF Pro2 data. Novel MoRE mode improved precursor detection and optimization of AIP settings, enhanced ion transmission. Up to 81,000 precursors and 7,700 protein groups were identified from 200 ng using dia-PASEF with a 30 ms ramp, while dda-PASEF with 300-Hz acquisition identified 37,000 precursors and 5,200 protein groups. These results match timsTOF Pro2's proteomics performance, while providing superior metabolomics and lipidomics capabilities, positioning it as a promising platform for scalable multi-omics research.

Glycoproteomics identifies disease-associated N-glycans and reduced occupancy of glycosylation sites in PMM2-CDG and MPI-CDG

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The diagnostic pathways of congenital disorders of glycosylation (CDG) often involves measuring analysis of carbohydrate-deficient transferrin (CDT). However, transferrin analysis selectively detects defects in N-linked glycosylation, accounting for only half of all CDG cases. Also, for certain CDG subtypes, such as PMM2-CDG, CDT testing may still yield false-negative results. Thus, there is a need for new more general biomarkers for CDGs, which enables detection of site-specific alterations in protein glycosylation, for accurate diagnosis, monitoring and effective stratification in clinical studies. With a LC-MS/MS glycoproteomic workflow that combines the analysis of N-glycopeptides and corresponding non-glycosylated peptides, we have analysed serum and plasma samples from individuals with well-characterized PMM2-CDG (n=7), MPI-CDG (n=2) and age-matched controls (n=9). We identified a glycopeptide from complement 3 with the oligomannose GlcNAc(2)Man(4) glycan at Asn-85 markedly elevated in PMM2- and MPI-CDGs. This finding also holds for the Asn-869 of alpha-2-macroglobulin in combination with the GlcNAc(2)Man(3) glycoform. For Asn-226 of complement 4-A/4-B, the GlcNAc(2)Man(5) glycoform was enriched in both patient groups. In addition, around 50 peptides with unoccupied N-glycosylation sites, including those of transferrin, were frequently detected in the PMM2- and MPI-CDG samples but not in controls. In summary, our glycoproteomic approach allows for identification of distinct CDG related panels of N-glycopeptides from various plasma glycoproteins, including the previously described xeno-tetrasaccharide N-glycan, and for recognition of widespread reduced N-glycosylation site occupancies. Our workflow has revealed promising biomarker candidates for the PMM2- and MPI-CDG glycophenotypes, correlating not only to CDG subtype but possibly also to disease severity.

Exploring P2-iST Plasma proteomics: From quantitative performance to CRC pilot study

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Human plasma is a rich source of disease biomarkers but remains difficult to analyze by mass spectrometry (MS) due to its extreme dynamic range and suppression by high-abundance proteins. We evaluated P2-iST Plasma, which combines P2 paramagnetic nanoparticle enrichment with iST-based sample preparation, and applied this workflow in a pilot study of colorectal cancer (CRC).

Quantitative performance was assessed using: (i) a seven-point species dilution series generated by mixing K2EDTA bovine and pooled K2EDTA human plasma (1:0 to 0:1; triplicates), and (ii) protein spike-ins (IL-6, IL-1β, MAPT) prepared as a five-fold serial dilution from 1250 ng to 0.4 ng in 100 µL human EDTA plasma. For the CRC pilot study, EDTA plasma from 12 donors (6 CRC, 6 healthy) was analyzed. Neat plasma and P2-iST Plasma samples (100 µL; KingFisher™ Flex) were measured using diaPASEF® on timsTOF HT coupled to Evosep™ One. Data was processed in Spectronaut® 20.

P2-iST Plasma expanded the detectable dynamic range to 7–8 orders of magnitude and increased the number of precisely quantified proteins (CV <20%) by 5–7-fold compared to neat plasma. Accurate fold-change behavior and robust linearity were maintained, with improved calibration-based LOD/LOQ in species mixtures and spike-ins. In the CRC pilot cohort, proteome coverage increased ~6-fold, revealing expanded CRC-associated signals and pathway enrichment. Correlation-based signatures centered on PTP4A3, and established plasma markers provided complementary discovery and quantitative reference points.

Overall, P2-iST Plasma substantially improves proteome depth while preserving quantitative accuracy, enhancing biomarker discovery in clinical plasma studies.

From aggressive clones to therapeutic vulnerabilities: proteomics maps mitochondrial states in melanoma

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Aggressive melanoma clones can persist through metabolic states hidden by bulk analyses. We asked whether mitochondrial activity defines a spatially organized and therapeutically actionable phenotype across melanoma progression.

We integrated quantitative proteomics from primary and metastatic melanoma; AI-guided digital pathology and spatial proteomics of primary melanomas with or without metastatic progression, metastatic lesions, and a longitudinal single-patient case; and mitochondrial proteome profiling in a large cohort. Mitochondrial dependencies were tested using inhibitors of mitochondrial translation and respiration.

Across datasets, aggressive, recurrent and metastatic melanoma converged on increased mitochondrial translation, oxidative phosphorylation and TCA-cycle activity, accompanied by reduced antigen-presentation and immune-response pathways. Spatial analysis localized this phenotype to tumor compartments associated with recurrence and an immune-suppressed stroma. In the longitudinal case, AI identified two morphologically distinct populations in the primary tumor. The population most similar to subsequent lung and brain metastases already displayed enhanced mitochondrial and glycolytic metabolism and MAPK signaling; the metastases further acquired PI3K–mTOR activity and SRC/YES1-associated resistance. In a larger cohort, mitochondrial activation intensified in BRAF V600E, advanced and treatment-resistant disease. Inhibiting mitochondrial translation or respiration reduced melanoma-cell proliferation, disrupted mitochondrial metabolism and induced apoptosis while largely sparing non-malignant melanocytes.

Proteomics identifies mitochondrial rewiring as a spatial cancer state linking metastatic competence, immune escape and treatment resistance. By resolving metastasis-competent tumor populations, these findings support patient stratification and nominate mitochondrial translation and respiration as biomarker-guided therapeutic vulnerabilities. The data support precision oncology strategies combining mitochondrial targeting with MAPK-pathway inhibition and immunotherapy in selected melanoma patients.

DDA and targeted glycopeptide characterization via hybrid collision- and electron-based dissociation strategies with timsOmniTM platform

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Glycopeptide analysis requires peptide identification, glycosylation site localization, and glycan composition assignment. However, glycan heterogeneity, limited fragmentation efficiency, and current data analysis tools constrain the comprehensive glycopeptide characterization. The timsOmni™ platform aims to address these challenges by combining trapped ion mobility with complementary dissociation methods. Here, we present a DDA-guided targeted-EXciD workflow that delivers enhanced analytical specificity while maintaining a high duty cycle.

Standard glycoprotein digest mixtures were analyzed using nanoElute™ LC separation combined with 1/K0–m/z polygon filtering on the timsOmni™. ExD and hybrid EXciD fragmentation methods were systematically optimized for both O- and N-glycopeptides. Non-targeted DDA parallel accumulation serial fragmentation (PASEF™) CID data were acquired and used to generate precursor inclusion lists via two strategies, either identifying glycopeptide precursors using MSFragger, GlycoScape™, and Byonic, or applying an in-house script for ranking CID precursors based on oxonium ion occurrence (number, intensity) and MS1 intensity to expand the target list, beyond the identifiable targets.

The workflow reproducibly triggered EXD events for up to 1000 targets with ~65% efficiency. EXciD fragmentation performance depended strongly on glycan size, precursor charge state, and ion mobility. ECciD outperformed for O-glycopeptides with smaller glycans and multiple glycosylation sites, generating extended backbone fragments preserving glycan moieties and facilitating localization. In contrast, EIciD was more effective for larger N-glycopeptides, increasing the number and size of B/Y fragments and facilitating glycan topology and structure determination. Additionally, ion mobility separation enabled resolution of glycan structural isomers. Manual annotation in OmniScape™ further increased confidence in complex EXciD spectral interpretation.

Longitudinal plasma proteomics of pregnancy reveals highly reproducible, trimester-resolved biology, paving the way for high-plex targeted absolute quantification with DiscoveryEdge600

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Here, we present a longitudinal pregnancy cohort study, publicly available through the Human Protein Atlas, and demonstrate a framework for large-scale quantitative plasma proteomics. A total of 300 plasma samples from 100 individuals were analysed across three trimesters using a Quantitative Recombinant Protein Standards (qRePS)-based workflow for absolute protein quantification with DiscoveryEdge175 on the Evosep One and Stellar mass spectrometer. qRePS are heavy isotope-labelled protein standards introduced prior to digestion, ensuring identical processing of standards and endogenous proteins and enabling process-controlled quantification.

The workflow enabled absolute quantification of 215 peptides representing 100 proteins, demonstrating high reproducibility across plates and replicates (median CV 7.5%). The longitudinal design allowed detection of biologically consistent, trimester-specific proteomic changes. Between the first and second trimester, 50 peptides corresponding to 23 proteins were significantly upregulated, while 86 peptides from 42 proteins increased in trimester 3, including complement and inflammatory proteins. In contrast, several core plasma proteins remained stable, and selected metabolic regulators decreased over time. Absolute quantification was critical for resolving physiological changes across time points without batch normalization.

Building on these results, we developed DiscoveryEdge600, a high-multiplex panel comprising 620 qRePS, enabling broad coverage of clinically and biologically relevant proteins involved in inflammation, cardiovascular, metabolic, coagulation and many more. The panel retains the same process-controlled quantification while extending the workflow to higher dimensional plasma profiling within a single standardized assay. Together, this work establishes a scalable strategy for absolute plasma proteomics, supporting biomarker discovery and validation.

10

Cleavage by different enzymes: analysis of tau protein in Alzheimer's disease brain tissue

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Background

Alzheimer's disease (AD) is characterised by two major brain changes: plaques of the amyloid beta (Abeta) peptide, and tau protein accumulations (tangles). Primary age-related tauopathy (PART) is a diagnostic classification for limited spread AD-like tau pathology, no Abeta pathology, and minor cognitive impairment. Detailed characterisation of tau is crucial to understand the molecular processes leading to tangle formation. Normally, trypsin used to produce peptides suitable for mass spectrometry (MS) analysis, cleaving C-terminally of lysine or arginine. However, not all parts of a protein can typically be monitored, which is also the case for tau. Using alternative enzymes, supplementary peptides can be monitored and contribute to a more complete molecular picture.

Method

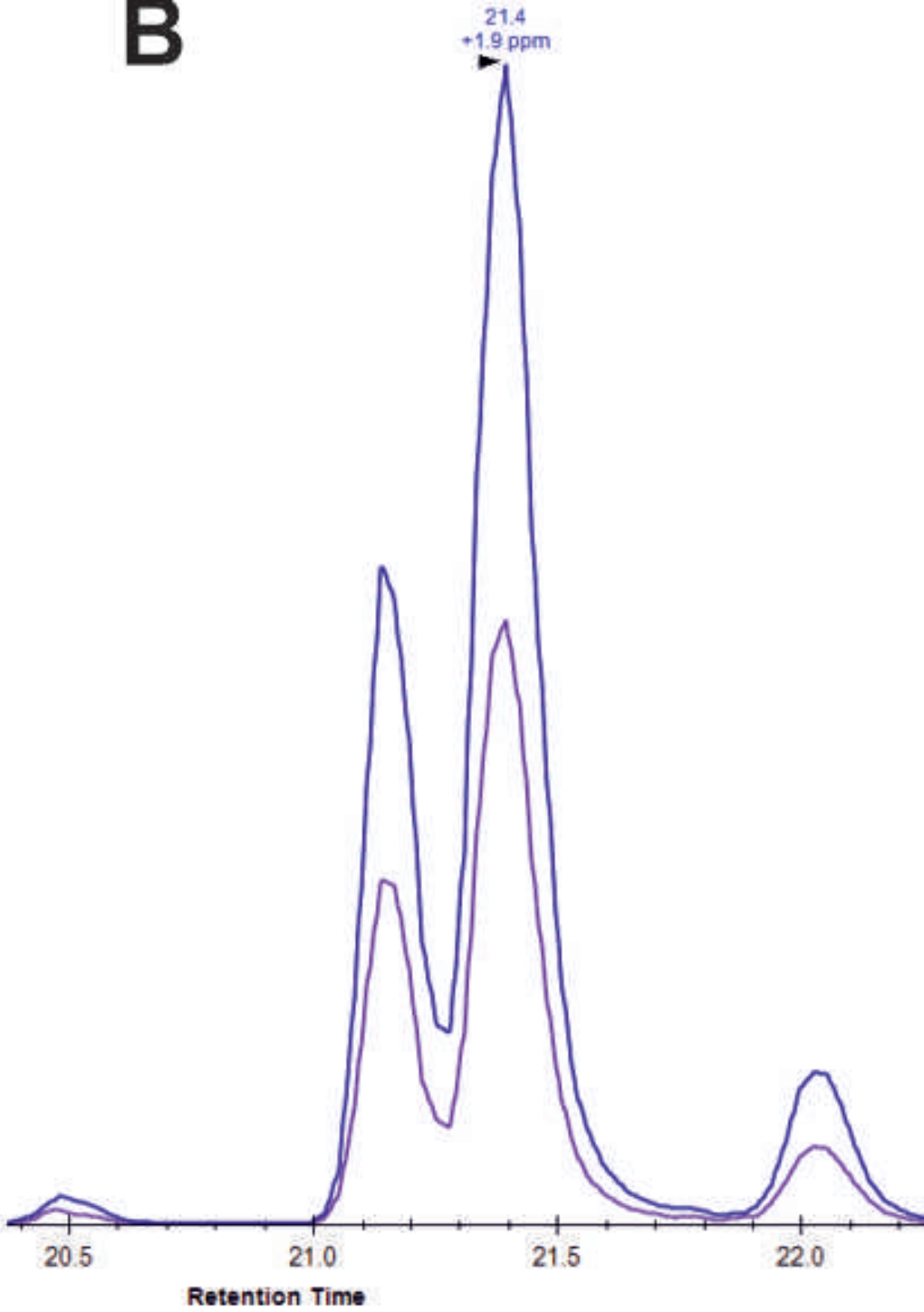
Tau from intermediate-AD, severe-AD and PART cases was analysed. Soluble (tris-buffered saline) and sarkosyl-insoluble brain extracts were immunoprecipitated and eluates digested with trypsin, LysC, and ArgC. Peptides were quantified by nanoflow LC/MS using isotope-labelled standards.

Results

The combined data from three different digestions leads to almost 100% coverage compared to 67% with trypsin. The main benefit, however, is the possibility to assess particular sequence parts or endogenous cleavages not possible to explore with trypsin alone. The approach is a powerful tool in characterization of particular proteins, e.g., tau. Examples of regions of interest are a group of peptides ranging from aa241-X, semi-digested by ArgC, and 376-383, digested by LysC, which can be deamidated at N381 and subsequently isomerised into variants of aspartate.

Conclusion

LysC and ArgC are very useful complements to trypsin for analysis of particular molecular features.



A) Semi-digested ArgC peptides 243-X. Peptides including aa 255-259 cannot be assessed with trypsin or LysC due to the lysines present but with ArgC it is possible.

B) Chromatographic trace of the four isomers of the 376-383 peptide deamidated at Asn381.

11

a masked variational autoencoder for sparse imaging mass spectrometry of parkinson's brain tissue

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Imaging mass spectrometry (IMS) provides spatially resolved molecular profiling of tissue sections. Downstream analysis remains difficult because the data are high-dimensional and sparse, vary between slides, and are hard to connect to interpretable biology when patterns are extracted without supervision. We present an analysis workflow built on a probabilistic variational autoencoder for sparse IMS data, developed to learn low-dimensional representations of human post-mortem brain tissue related to Parkinson's disease. The model combines peak intensities with an explicit observation mask, reconstructing measured ion intensities while modeling missingness separately rather than treating absent peaks as zero. We apply the method to FMP-10-labeled features from striatum and substantia nigra sections to identify tissue-region structure and peaks associated with dopaminergic and serotonergic pathways. Preliminary results indicate that the latent space captures spatial and sample-level structure, and that diagnostic plots identify the molecular features driving the major latent axes. The workflow thus separates technical effects from biological variation and supports interpretation of sparse IMS data.

a scalable nextflow pipeline for analysis and evaluation of targeted proteomics

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Targeted proteomics enables sensitive and reproducible quantification of predefined target proteins or peptides. The current analysis is now fragmented across method-specific tools, which is not able to scale with large cohorts. Besides, it is rather limited in statistical error control tailored to targeted data.

We are developing TEAPOT (Targeted Evaluation and Analysis Pipeline for Proteomics on Tandem mass spectrometry), a Nextflow pipeline intended to automatically perform high-throughput targeted proteomics analysis with a modular infrastructure. The schematic overview of the pipeline is illustrated in the attached flowchart. From an SDRF-defined input, it performs format conversion (msconvert), spectral and chromatogram library preparation, and targeted signal extraction and feature scoring using EncyclopeDIA2, OpenSWATH, DIA-NN, or Skyline. PRM features are rescored in Context (integrating mProphet and Percolator) and DIA features in Percolator or pyProphet; pyIsoPEP estimates q-values and monotone posterior error probabilities; and diathem harmonizes cross-run quantification via intensity-ratio normalization. Context, pyIsoPEP, and diathem are developed in-house, and quality-control reporting is being added.

By providing a comprehensive analysis workflow, TEAPOT aims to deliver a reproducible, statistically controlled, and scalable framework for targeted proteomics analysis. We present the current architecture and discuss the design choices behind its statistical error control.

A melanoma physician's journey: from patient care to molecular understanding

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As physicians, we are driven to improve patient outcomes. By implementing advanced proteomics and molecular profiling with AI-driven tumor morphology in our research laboratories, we aim to uncover the biological pathways governing melanoma progression and therapeutic response, translating these insights into informed clinical decisions.

In addition, melanoma increasingly becomes a long-term disease to manage, it carries a socioeconomic burden extending beyond the patient to families and caregivers. Disease courses still defy expectations, such as early-stage presentations that progress silently, making individualized risk stratification not a future ambition but a present necessity.

This is why we pursue one of the largest melanoma studies combining quantitative proteomics with AI-based software for identifying tumor regions and cellular populations: morphology and molecular function are too often read as separate languages by different specialists at different stages of care. We follow each patient from primary melanoma through metastasis, correlating these layers with clinical metadata to guide therapy choice.

Our approach computationally correlates AI-derived morphological features with proteomic signatures from the same tumor regions. Using QuPath, whole-slide images are tiled and segmented via a trained nnU-Net model into tumor, stroma, necrosis, and immune compartments, then linked to molecular data from corresponding regions, so pathology and mass spectrometry become two views of the same biology.

Behind every segmented tile lies a clinician's question: what does this tell us about this patient's journey to metastasis? Grounding our approach in morphology and molecular function, and following each patient individually, we strive for results focused on patient well-being.

exploring protein-based stratification in early alzheimer's disease using discovery and targeted proteomics

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Background: This study aimed to characterize plasma proteomic changes and protein-based patient stratification across the Alzheimer's disease continuum using high-throughput and targeted proteomics approaches.

Materials and methods: Plasma samples from 281 participants in the Wisconsin Registry for Alzheimer's Prevention cohort were stratified into healthy (A−T−), early AD (A+T−), and later-stage AD (A+T+) groups according to cerebrospinal fluid biomarkers. High-throughput plasma proteomics was performed using Olink Explore HT Proximity Extension Assay (PEA, ~5,400 proteins) and targeted liquid chromatography–tandem mass spectrometry (LC–MS/MS). Differential abundance analysis, weighted gene co-expression network analysis (WGCNA), unsupervised clustering, gene set enrichment analysis, and differential correlation network analysis were applied.

Results: Differential abundance analysis identified five progression-associated proteins. GFAP and CPN2 showed progressive increases, whereas PODXL, CCL28, and SERPINE1 showed consistent decreases across disease stages. WGCNA and unsupervised clustering identified two major proteomic subtypes characterized by either intracellular biosynthetic activity or inflammatory and structural remodeling. Integrative network analysis revealed extensive regulatory rewiring involving the master hub proteins ITGAM, CXCL10, ERBB2, and TNF, indicating a transition from metabolic stress in early disease to immune and proteostatic stress in later stages. Targeted LC–MS/MS analysis showed generally modest fold changes, with only two peptides remaining significantly altered after multiple testing correction.

Conclusion: Plasma proteomics captures stage-specific biological networks and molecular heterogeneity across the AD continuum and identifies candidate biomarkers and network hubs that may support biology-driven blood diagnostics and patient stratification in Alzheimer's disease

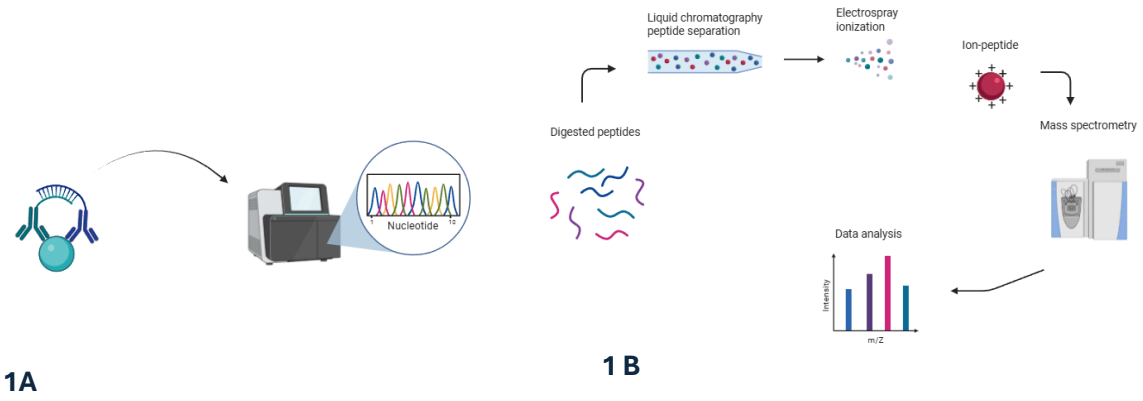
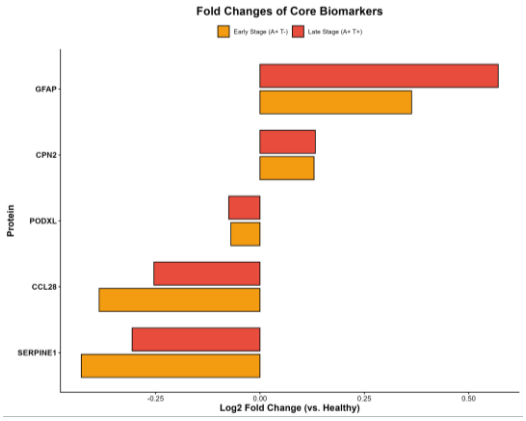
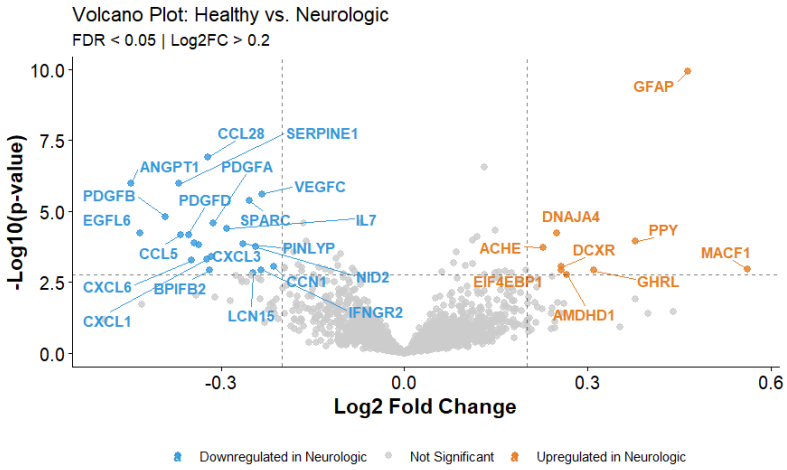


FIGURE 1 ILLUSTRATION OF HOW OLINK AND MASS SPECTROMETRY METHOD WORKS.

A) Olink & B) Mass spectrometry





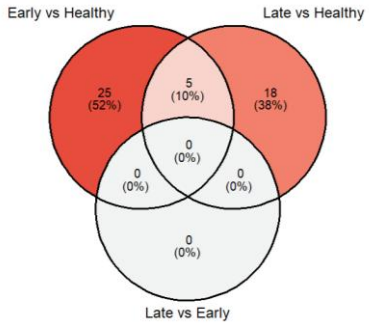


Figure 2. Differential abundance analysis

Volcano plot showing differential protein abunance between healthy controls and neurologic disease groups. Significantly upregulated proteins are shown in orange and downregulated proteins in blue (FDR < 0.05, |Log2FC| > 0.2). Venn diagram showing overlaps of differentially expressed proteins across Early vs. Healthy, Late vs. Healthy, and Late vs. Early comparisons. Five proteins were shared between the Early and Late disease stages, while no significant proteins were identified directly between the Early and Late stages. Comparison of Log2 fold changes for the five overlapping proteins in Early and Late disease stages relative to healthy controls.

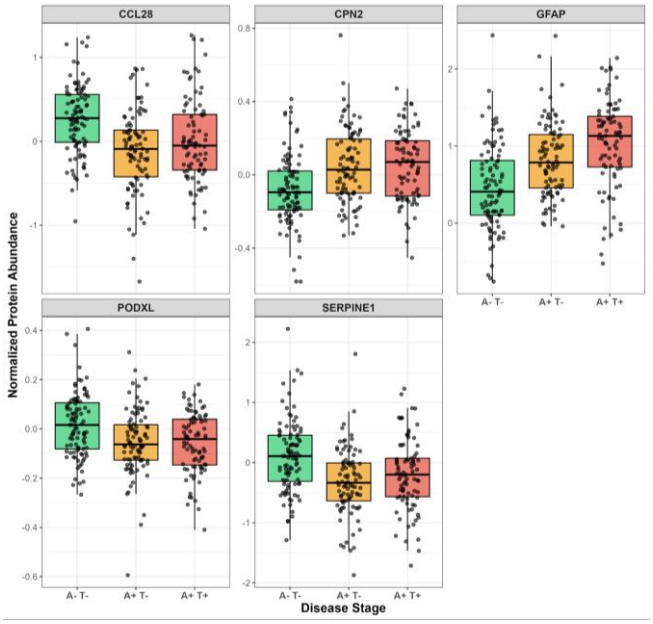


Figure 3. Normalized protein abundance of core biomarkers across disease stages.

Boxplots showing normalized abundance levels of the five overlapping biomarkers (CCL28, CPN2, GFAP, PODXL, and SERPINE1) across healthy controls (A−T−), early-stage disease (A+T−), and late-stage disease (A+T+). Each point represents an individual sample. GFAP and CPN2 showed increased abundance across disease progression, while CCL28, PODXL, and SERPINE1 showed reduced abundance in the disease groups compared with healthy controls.

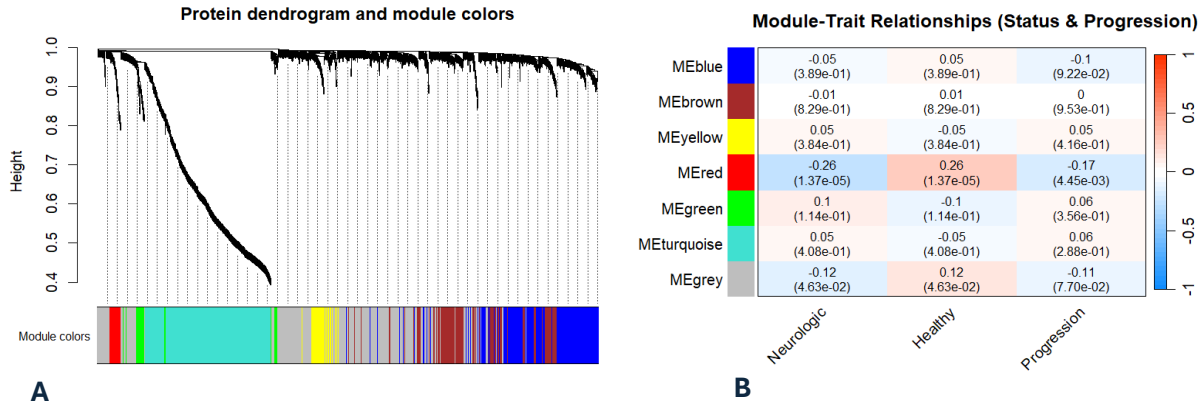


Figure 4. WGCNA modules and their association with disease traits.

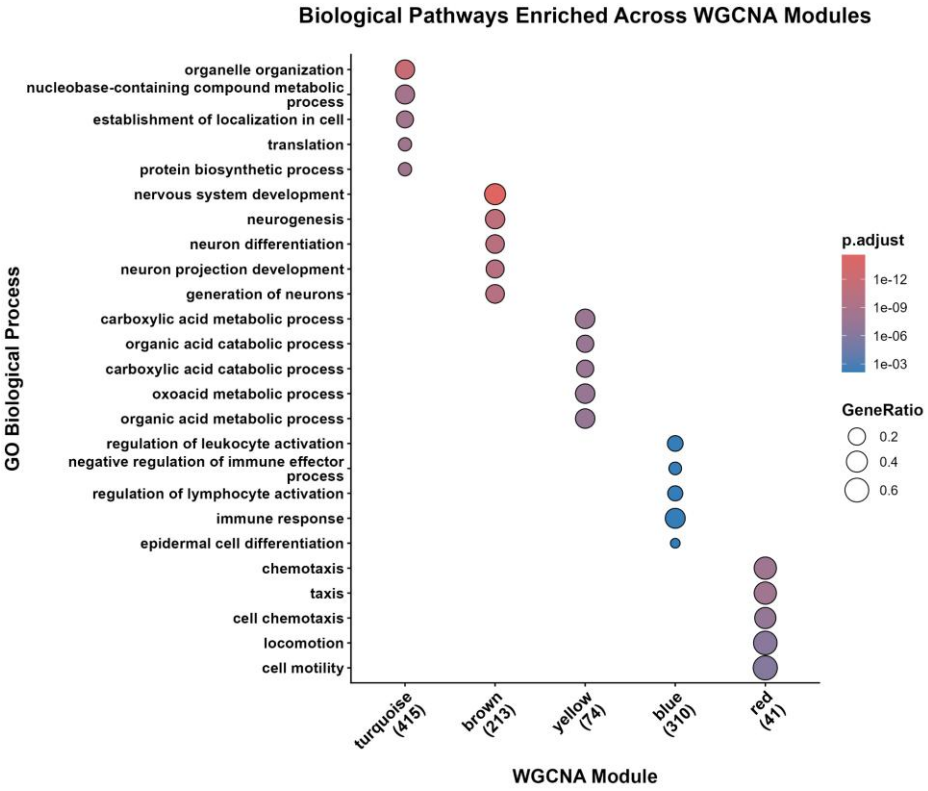


Figure 5 Gene Ontology enrichment analysis of the co-expression modules

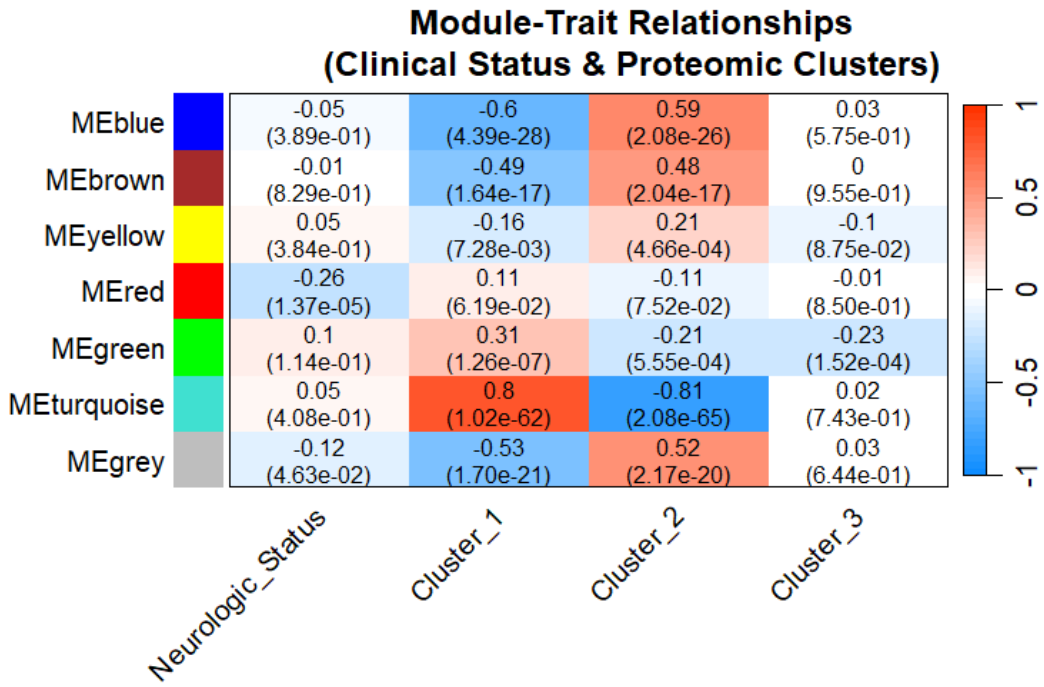


Figure 6. Module-trait relationships across proteomic patient subtypes.

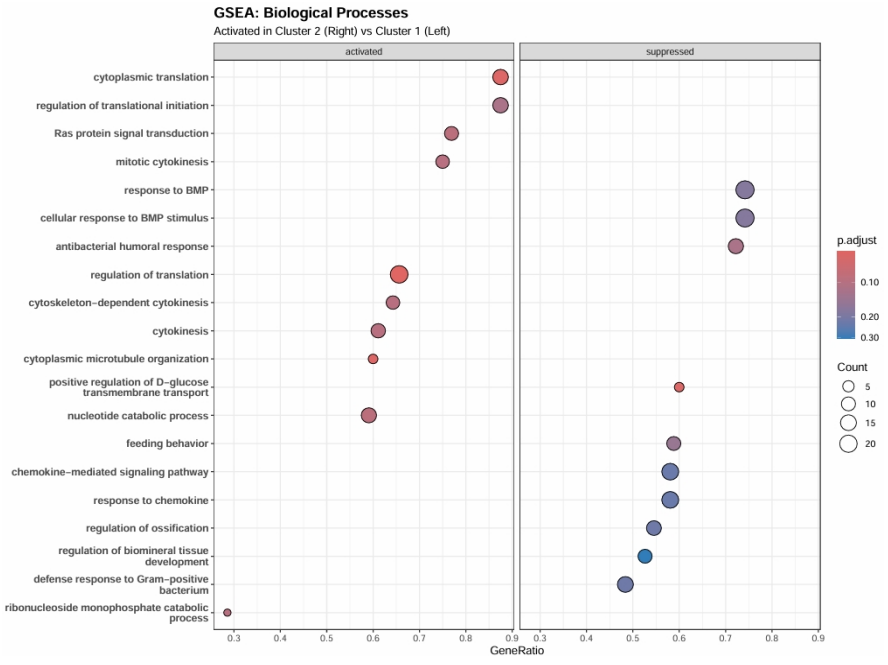


Figure.7 Gene set enrichment analysis.

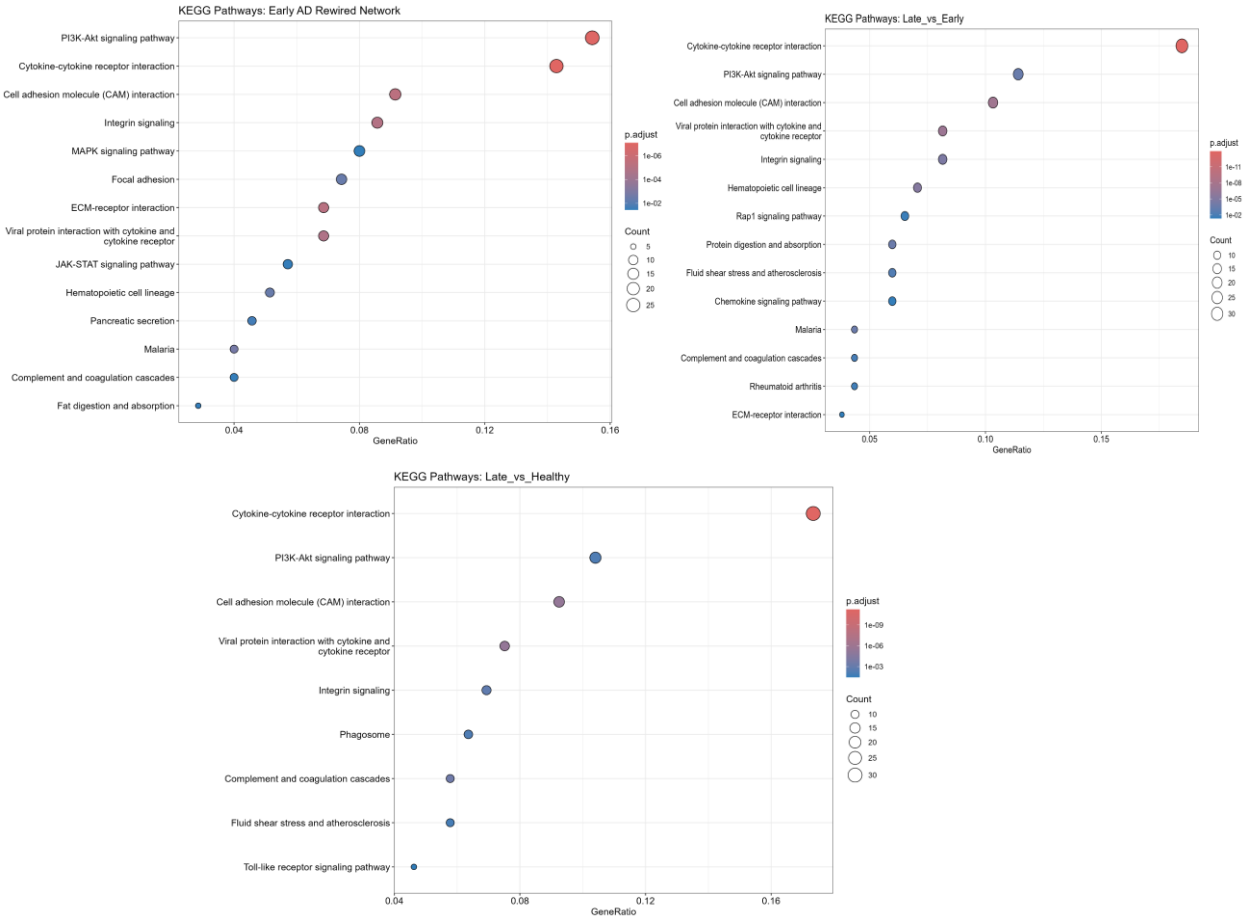


Figure 8. KEGG pathway enrichment of high-connectivity network hub proteins.

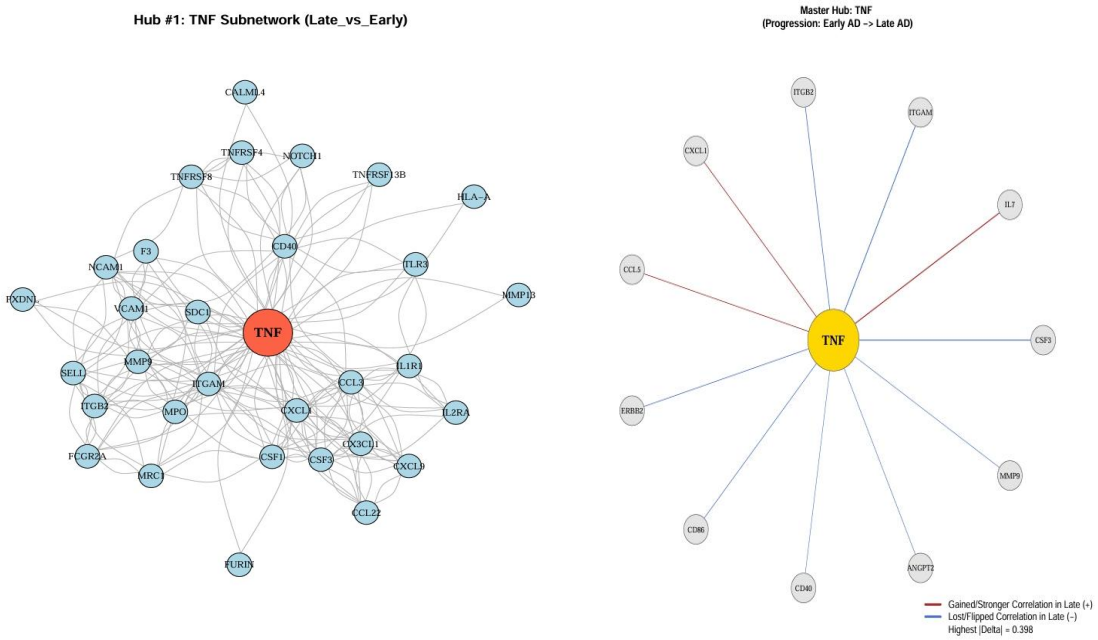


Figure 9. Integrative network analysis of the TNF hub during disease progression.

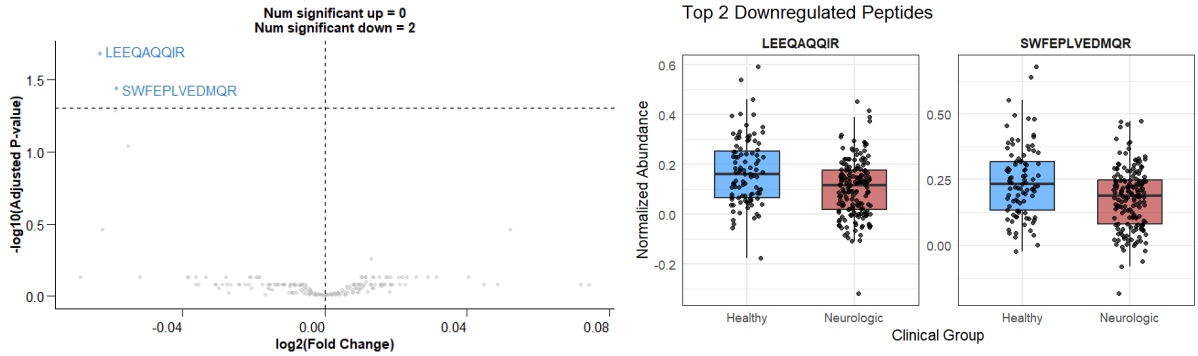


Figure 10. Differential abundance analysis after multiple testing correction and the subsequent boxplot

Mass Spectrometry and Bioinformatics-Assisted Discovery of fungal Microproteins

Ram Anup [7, 8], Gonzalez Jose Antonio Flores [7, 8], Morgan Jared [7, 8], Stakaitis Rytis [7, 8], Aramburu Iker Valle [7, 8]

[7] 1Department of Molecular Biology, Umeå University, Umeå, Sweden., [8] 2Laboratory for Molecular Infection Medicine Sweden (MIMS) and Umeå Center for Microbial Research (UCMR), Umeå University, Umeå, Sweden.

Background: Microproteins, or small open reading frame (sORF)-encoded polypeptides (SEPs), are translated peptides typically smaller than 150 amino acids. Although historically overlooked, recent finding and technological advancements are producing compelling evidence of translation occurring beyond the known annotated open reading frames (ORFs)1. In this study, we aim to identify sORFs with coding potential in the commensal pathogenic fungi Candida albicans under homeostasis and upon host-pathogen interaction using mass spectrometry. Identifying novel microproteins by mass spectrometry is hindered by the absence of a reference database and the limited number of peptides generated from their small size.

Material and methods: We have built a comprehensive database of potential sORFs by doing 6-frame translation of C. albicans genome, which yielded >1M sORF. We applied various filtering strategies, including the previously published DBReducer method, to reduce the database size while maintaining control over the FDR. We also did de-novo peptide sequencing using InstaNovo to discover peptides independent of a database. Additionally, we tried different enrichment and MS acquisition strategies to enrich microproteins and improve our outcome.

Results: By applying all these aforementioned strategies, we have managed to discover close to 100 novel microproteins in C. albicans in yeast, hyphal form or when co-cultured with neutrophils.

Conclusion: This study has helped us illuminate the novel microproteome in C. albicans which can play a critical role in fungal growth, morphology switch and fungal pathogenicity.

Arboviral Antibody Profiles in a Pediatric Cohort of suspected Central Nervous System Infections in southwestern Uganda

Sosa Acosta Patricia [2, 3], Gomes da Silva Priscilla [3], Mlotshwa Phuthumani [4], Gardell Anna [3], Skoglund Lovisa [3], Kumbakumba Elias [5], Mwanga-Amumpaire Juliet [5], Gaudenzi Giulia [4], Nilsson Peter [2, 3]

[2] SciLifeLab, [3] KTH Royal Institute of Technology, [4] Karolinska Institutet, [5] Mbarara University of Science and Technology

Central nervous system (CNS) infections are a major global public health concern, with mortality rates of up to 50%. The aetiology of CNS infections spans diverse pathogens and often remains undetected in African countries. Recent studies from a pediatric suspected CNS infection cohort showed a higher prevalence of malaria (20%) and other bacterial pathogens (11%), while no pathogen was identified in 58% of cases. Although Dengue seroprevalence is generally low in African children, the contribution of arboviral infections to CNS disease remains unclear. Here we aim to identify and quantify arboviral antibody profiles in children admitted at two hospitals in Uganda with suspected CNS infections across four age groups.

Forty viral antigens were immobilized onto color-coded magnetic beads, including antigens from common influenza viruses and several arboviruses. Antigen-coupled beads were pooled and incubated with diluted serum samples obtained from control patients with febrile illness and patients with CNS infection. Antibody abundance was measured using the FlexMap 3D instrument.

We identified age-dependent differences in flavivirus antibody abundance profiles. Antibody profiles in newborns and children differed from those observed in infants and young children. In contrast, infants and young children showed greater dispersion in flavivirus antibody profiles, suggesting more dynamic pathogen exposure during these stages of life. Antibodies against DENV-NS1 antigens were the main drivers of these age-specific antibody patterns.

This study could enhance our understanding of how flavivirus antibodies are transferred from mother to child, how antibodies develop throughout childhood, and the role tropical viruses play in causing pediatric CNS infections.

17

chemical proteomics meets structural proteomics: marriage or crash?

Zubarev Roman [1]

[1] Karolinska Institutet

Structural proteomics has been struggling to reach truly system-wide dimension. Cross-linking captures tiny bit of structural information present in the proteome. Hydrogen-deuterium exchange mass spectrometry yields details on structural motifs and binding sites of isolated molecules, but scaling up to whole proteome is problematic. Labelling techniques complicate the analysis and require two different peptides to be quantified, which limits the scope to high-occupancy cases. Recently, we developed a technique that could live up to the "structural proteomics" label. In HigH ratiO partiaL proteolysiS with carriER proteome (HOLSER), partially digested proteome is multiplexed using tandem mass tag (TMT) with fully digested proteome (Zhang X. et al., Analyt. Chem, 2026, in press). The ratio Rpf between the reporter ions in the same TMT set representing partial and full digests is the protease accessibility factor that ranges between zero for peptides buried inside to unity for surface peptides. Thus Rpfs provide structural information, identifying the drug target and the binding site. In a HOLSER experiment, up to 150,000 peptides are quantified, each one with Rpf, with on average ≥20 Prfs per protein. Using this information, one can deduce the structure of 5,000-8,000 proteins simultaneously, while full digest profiles expression levels. As TMT-18 can multiplex the full digest and five cell states, in triplicate each, a single analysis can cover the whole study. The use of AI for deriving protein structure using both sequence and Rpfs and as constraints, the necessity of developing the "motif search engine" and HOLSER's application field are discussed.

krysin, a high-fidelity protease for plasma proteomics and post-translational modification analysis

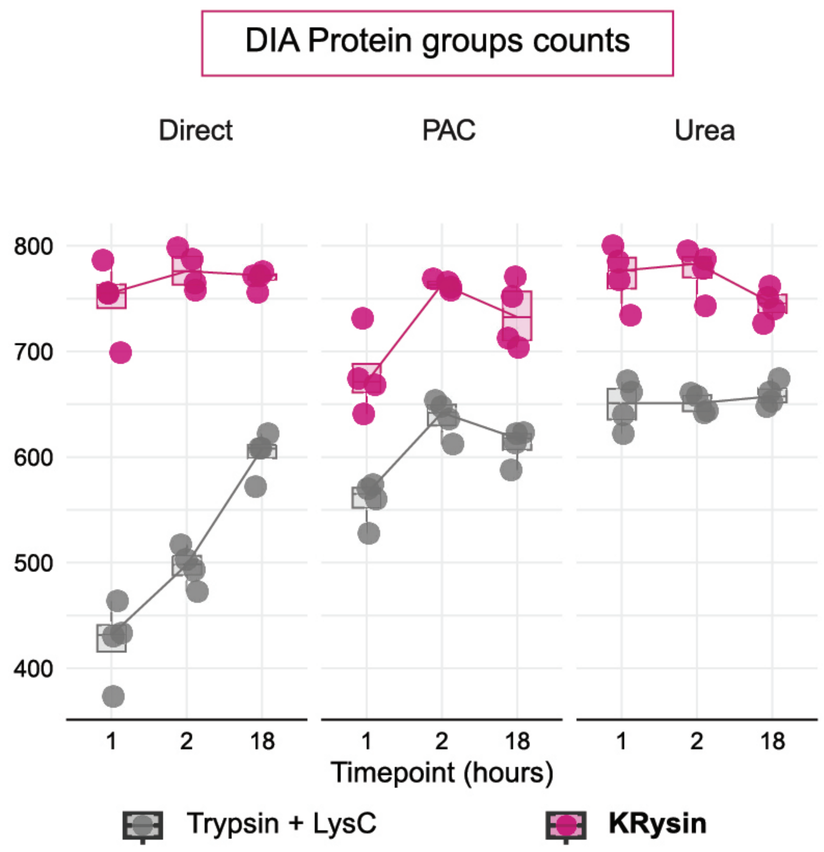
Hernandez Rollan Cristina [1], Koenig Claire [2], Batth Tanveer Singh [1], Olsen Jesper Velgaard [2]

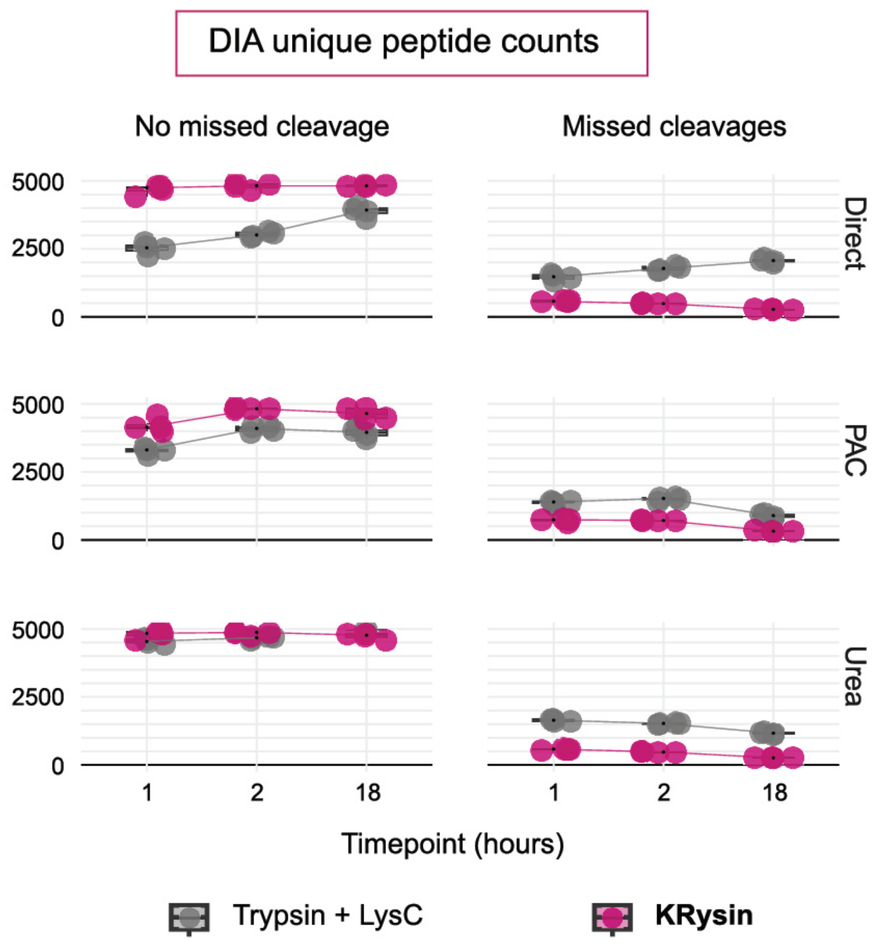
[1] KPL ApS, [2] University of Copenhagen

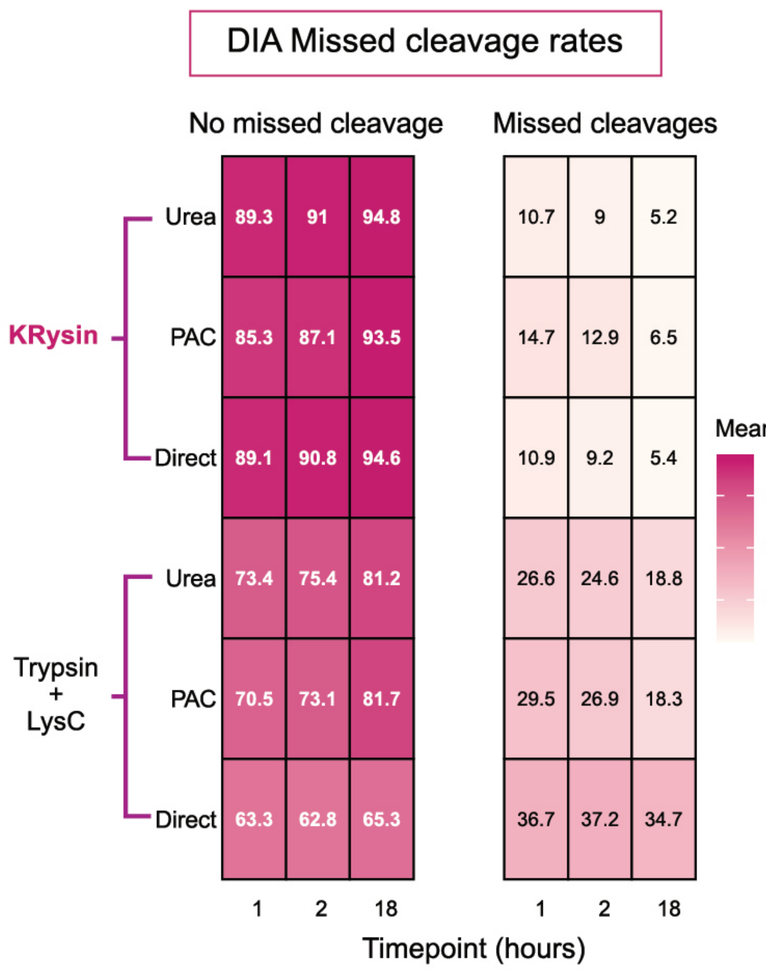
Conventional proteases such as Trypsin and LysC underperform in the most demanding proteomics applications. In plasma, endogenous protease inhibitors and a high dynamic range cause missed cleavages and incomplete digestion. In post-translational modification (PTM) analysis, phosphorylation near Lysine or Arginine residues creates local acidity and salt bridges that reduce cleavage efficiency, leaving many sites undetected.

We developed KRysin, a high-fidelity protease that outperforms conventional proteases across both contexts. For plasma proteomics, platelet-depleted plasma was digested across multiple preparation methods and digestion times. For phosphoproteomics, HeLa lysates were digested with Trypsin, Trypsin/LysC, or KRysin and analysed by narrow-window data-independent acquisition on an Orbitrap Astral. In plasma, KRysin achieved 94-98% of peptides with zero missed cleavages, a 20% increase in protein groups (up to 775), and four- to fivefold higher signal intensity. Time-course analysis showed near-optimal digestion within one hour. For phosphoproteomics, KRysin yielded 49,888 class I phosphosites per file, at least 30% more than conventional proteases, with 80% of phosphosites fully cleaved and superior recovery of multiply phosphorylated peptides in acidic microenvironments. Notably, KRysin, at a low enzyme-to-protein ratio, outperformed Trypsin/LysC used at three times the amount. Together, these results establish KRysin as a versatile high-efficiency protease that overcomes biochemical barriers across PTM and plasma workflows, enabling comprehensive site discovery and deeper proteome coverage.

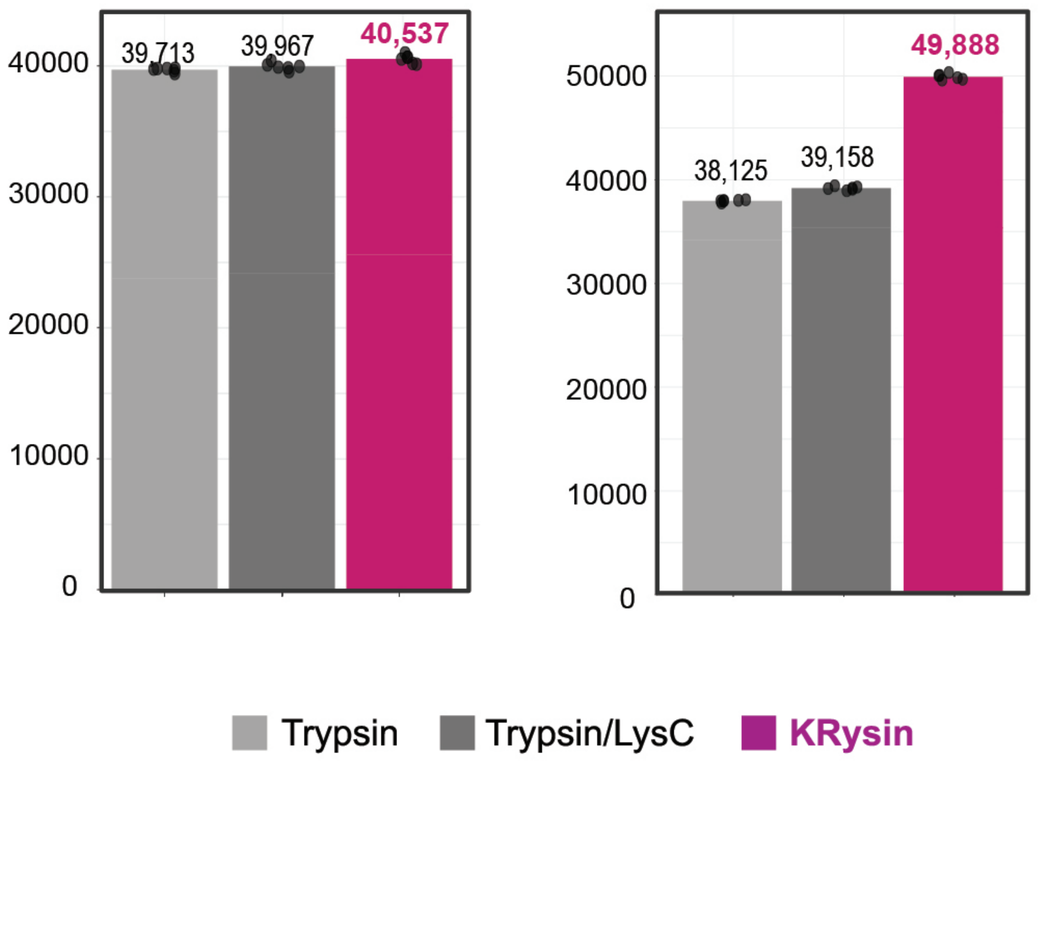
Plasma proteomics

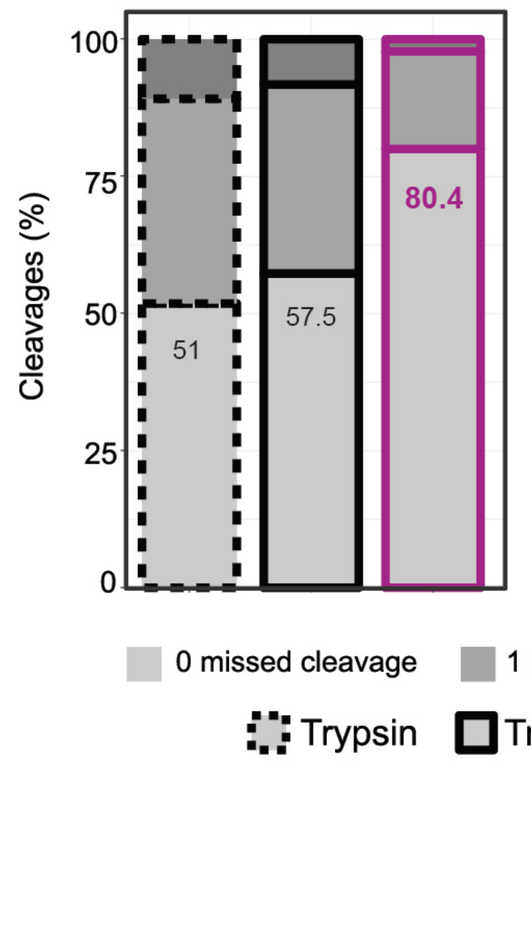


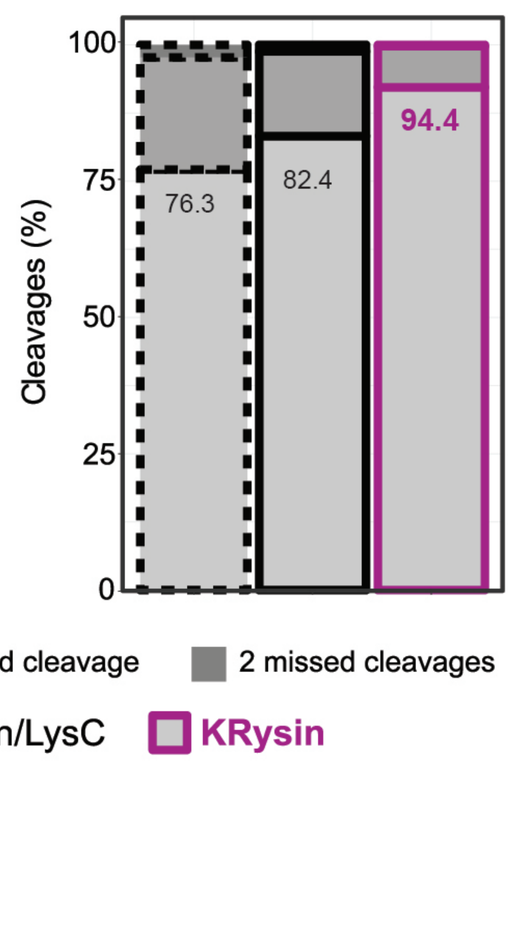




Phosphoproteomics







BeatBox® and SP3®-iST enable scalable, high-throughput sample preparation for fecal metaproteomics

Moritz Chloé [2], Tanca Alessandro [1], Ravi Kumar Ranjith Kumar [3], Xian Feng [3], Deledda Maria Antonietta [1], De Diego Laura [1], Abbondio Marcello [1], Uzzau Sergio [1], Gomez-Varela David [3]

[2] PreOmics GmbH (part of Biognosys Group), Martinsried, Germany, [1] Department of Biomedical Sciences, University of Sassari, Sassari, Italy, [3] University of Vienna-Bruker Center of Excellence for Metaproteomics, Vienna, Austria

Fecal metaproteomics enables functional characterization of gut microbiota and host responses but remains analytically challenging due to the complexity of the fecal matrix and the need for efficient, reproducible, and scalable sample preparation. Here, we present two independent advances addressing these challenges: BeatBox for standardized fecal homogenization and SP3-iST for high-performance protein extraction, cleanup and digestion.

BeatBox was evaluated for parallel processing of mouse fecal samples in a multiwell format. The extracted proteins were reduced, alkylated, cleaned, and digested into peptides, followed by LC–MS/MS-based metaproteomic analysis, database-driven taxonomic and functional annotation.

Cleanup and digestion workflows for fecal metaproteomics were benchmarked in low- and high-throughput formats. Automatable approaches based on solid-phase and filter-based principles were compared using peptide identifications, digestion efficiency, physicochemical peptide coverage, and quantitative reproducibility as performance metrics.

BeatBox homogenization enabled reproducible protein extraction from 5 mg mouse fecal samples in batches of up to 96, yielding ~10,000–15,000 microbiota-derived and ~2,700–3,600 host-derived peptides per replicate. This supported annotation of nearly 50 microbial species and >140 KEGG pathways.

SP3-iST achieved the highest peptide yields among tube-based methods for human feces (~7,500 peptides) and superior digestion efficiency, with up to 90% of peptides free of missed cleavages. Comparable performance was maintained in a 96-well format.

BeatBox and SP3-iST address distinct but complementary steps in fecal metaproteomics, together providing a complete, scalable and quantitative foundation for standardized, high-throughput microbiome proteomics.

20

Proteome-wide analysis reveals host responses to SpnA-mediated streptococcal pathogenesis

Panella Silvia [2], Neumann Ariane , Ertmane Elizabete , Frick Inga-Maria , Björck Lars , Happonen Lotta [2]

[2] Lund University

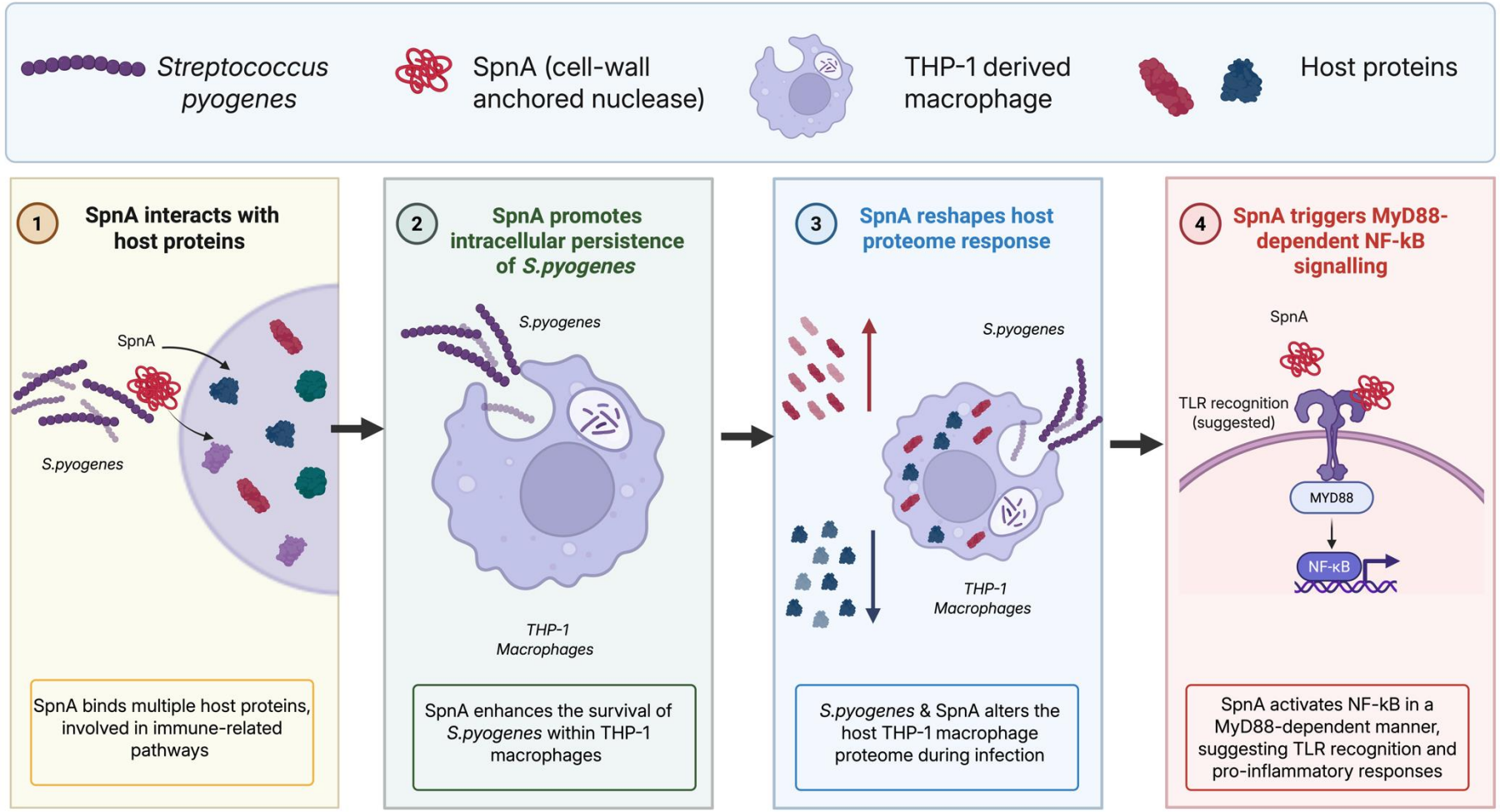
Background: Streptococcus pyogenes is a major human pathogen that expresses multiple virulence factors to evade host immunity. Among them, streptococcal nuclease A (SpnA) is a cell wall-anchored nuclease that degrades neutrophil extracellular traps (NETs) and inhibits the complement system membrane attack complex (MAC) formation. However, the cellular host proteins targeted by SpnA and the biological implications of these interactions remain uncharacterized.

Materials and methods: The cellular host interactome of SpnA was investigated using THP-1 cells and affinity-pulldown coupled to quantitative mass spectrometry (AP-MS). Intracellular bacterial adaptation and proteome-wide host responses to infection were assessed using data-independent acquisition (DIA) MS by infecting THP-1 derived macrophages with wild type and delta SpnA S. pyogenes strains. SpnA-induced NF-kappaB activation was evaluated using THP-1 reporter cells.

Results: Proteomic analysis identified multiple SpnA-interacting host proteins associated with immune-related pathways, with 104 interactors detected for the SpnA N-terminus and 102 for its core regions. Infection experiments demonstrated that SpnA promotes intracellular persistence of S. pyogenes. Proteome-wide analysis revealed distinct temporal host responses between 3 and 24 hours post-infection, including SpnA dependent differences in host protein regulation. Furthermore, SpnA induced NF-kappaB activation in a MyD88-dependent manner, supporting recognition through Toll-like receptor (TLR) signaling.

Conclusion: Our findings reveal previously uncharacterized interactions between SpnA and cellular host pathways and suggest that SpnA contributes to streptococcal pathogenesis by promoting intracellular persistence while modulating innate immune responses.

SpnA shapes host responses during *Streptococcus pyogenes* infection



SpnA is a key virulence factor that interacts with host proteins, promotes intracellular persistence of *S. pyogenes*, reshapes macrophage responses, and activates MyD88-dependent NF-κB signaling to facilitate streptococcal pathogenesis.

21

high□throughput single□cell multi□omics using standard□flow lc and a multi□reflecting tofms

Savolainen Otto [1], Kattla Jayesh [1], Daly Matthew [1], Gethings Lee [2], Lock Richard [1], Heywood David [1]

[1] Waters, [2] Wates

Single□cell lipidomics and metabolomics are increasingly important for uncovering cell□to□cell biochemical heterogeneity often masked in bulk analyses. However, extremely low analyte abundance in individual cells necessitates highly sensitive and robust analytical workflows. HT29 and CaCO2 gastrointestinal and cancer research cell lines were used to demonstrate a unified LC□MS strategy for high□throughput discovery profiling.

Single cells were isolated using the IsoPick microfluidic platform and deposited directly into LC vials. Lipids were extracted with ice□cold isopropanol containing deuterated internal standards, while polar metabolites were extracted with 0.1% formic acid using a validated method. Extracts were analysed on a U(H)PLC system equipped with a 2.1×100 mm phenyl□hexyl column operated at 0.4 mL/min and 70 °C, coupled to a multi□reflecting TOF mass spectrometer running DIA and DDA modes with enhanced duty cycle (EDC).

Processed datasets (LipoStar for lipids, MARS for metabolites) produced more than 200 lipid identifications and over 150 polar metabolites per single cell (n=5), spanning amino acids, organic acids and nucleotides. High□resolution MS1 and MS2 data, boosted by EDC for ~10× higher MS2 signal, improved confidence in structural elucidation. The phenyl□hexyl chemistry eliminated isopropanol from the mobile phase, reducing background signal and enhancing signal□to□noise.

This streamlined LC□MS configuration enables comprehensive single□cell lipidomics and metabolomics without hardware changes, providing an efficient and scalable platform for multi□OMIC discovery studies.

22

Multiomics of fecal extracts

Kattla Jayesh [1], Savolainen Otto [1], Daly Matthew [1], Gethings Lee [1], Lock Richard [1], Heywood David [1]

[1] Waters

Fasiglifam (TAK□875) is an orally bioavailable, selective GPR40/FFAR1 agonist developed for Type 2 diabetes, promoting glucose□dependent insulin release. Although early dose□finding studies showed reduced hypoglycemia risk, development stopped after Phase III trials due to liver toxicity concerns. Previous metabolism studies indicated that most TAK□875 metabolites are eliminated in feces, and growing evidence shows bidirectional interactions between drugs and the gut microbiome that can influence drug efficacy. This study applied a multiomic strategy to explore TAK□875–related microbiome and biological changes in a rat model.

The study used 15 male and 9 female Sprague Dawley rats, initially pair-housed then singly housed for a 96□hour study duration. Animals received fasiglifam intravenously (5 mg/kg) or orally (10 or 50 mg/kg), with vehicle controls for both routes. Fecal and biological fluid samples were collected from predose to 96 hours. Fecal samples were extracted for polar metabolites (Folch) and proteins, which were reduced, alkylated, and digested using FASP.

Metabolite extracts were analyzed using a 5□minute HILIC□UHPLC method coupled to a multi□reflecting Q□TOFMS. Proteomic datasets used the same MS with capillary LC, acquired using DDA and DIA with Enhanced Duty Cycle to improve MS2 sensitivity and sequence coverage. Proteomic searches used curated FASTA databases combining rat (Uniprot) and gut□microbial protein sequences, informed by shotgun metagenomics.

Integrated proteomic and metabolomic analyses revealed coordinated dysregulation across the time course, aligning with TAK□875 pharmacometabodynamics. Notably, expression of G proteins such as GPR84 and GPR43 increased, suggesting altered regulation of medium□chain fatty acid signaling and immune□related short□chain fatty acid pathways.

Chemical Protoemics, a unique unit of BioMS and SciLifeLab at Karolinska Insitutet for MoA characterization

Gaetani Massimiliano [1, 2, 3]

[1] Karolinksa Institutet, [2] Scilifelab, [3] BioMS

The Chemical Proteomics unit provides proteome-wide chemical proteomics support to identify drug targets and decipher mechanisms of action of any bioactive molecules perturbing any biological systems.

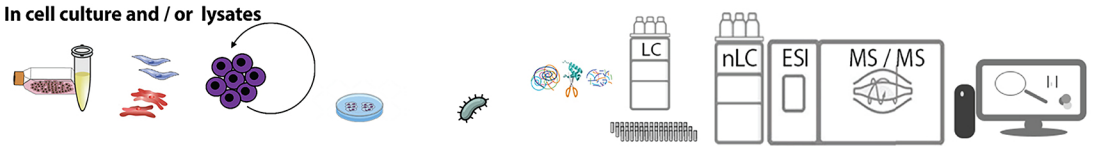
Using the PISA assay, we deconvolute targets and early molecular responses through solubility shifts measured in a full proteome profiling mode. Complementarily, HOLSER, also reaching deep proteome profiling, enables global structural profiling and site-resolved characterization of ligand–protein interactions, providing mechanistic insight into binding interfaces and conformational rearrangements.

Our platform integrates multiple proteomics modalities—including expression, PTM, and RedOx profiling, as well as HDX-MS for structural characterization—within end-to-end workflows from experimental design to data interpretation.

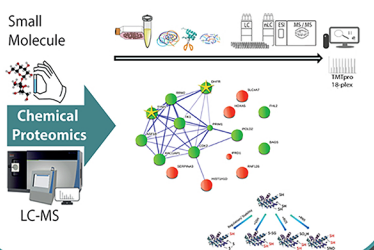
Projects are co-designed in close scientific dialogue and executed through high-quality workflows—from controlled cell or lysate treatments to deep, unbiased LC–MS-based proteome profiling—ensuring robust and interpretable mechanistic insights into compound action.

Projects can be also integrated and coordinated beyond proteomics at the CBGE platform level for multi-modal data-driven MoA elucidation.

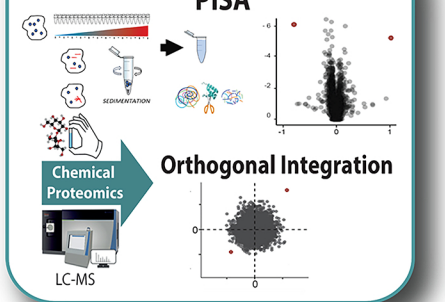
Target Deconvolution and MoA characterization through multimodal full proteome profiling approaches (*e.g.*, post phenotypic screening or assays)



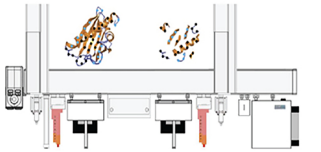
Deep Proteomics Profiling Expression / PTMs /RedOx

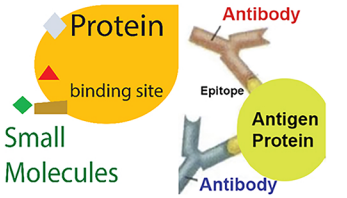


Target ID and MoA PISA

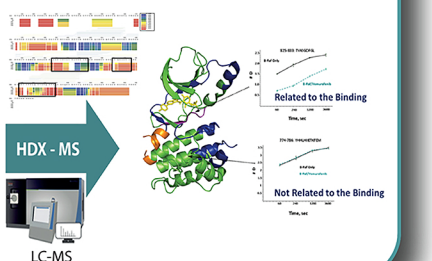


HDX-MS for Binding Site and Conformational Change Mapping





Target Binding Site Mapping HDX-MS



Binding site mapping
- Protein-peptide
- Epitope mapping (Ag-Ab)
- Protein-small molecule

Conformational changes
- Due to mutation
- Misfolding
- Allosteric effects

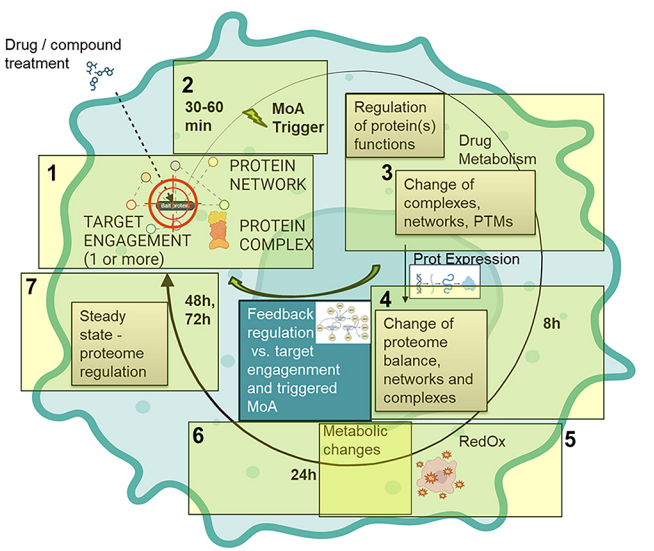
Cellular proteome

- *High protein density in cells: e.g.,* 200–300 mg/mL in the cytoplasm
- *Molecular Crowding:* high protein density affecting biochemical reactions, diffusion, protein folding and macromolecular assembly
- *Dynamic range of intracellular protein abundance and concentrations:*
- 6 to 7 orders of magnitude

Our chemical proteomics approaches to decipher direct targets, induced mechanisms and cell proteome responses

thorugh multi-modal, quantititative, multiplexing, proteome-wide and deep proteome profiling (up to 10 thousands proteins or more reliably identified and quantified, large sample multiplexing for ≠ conditions, cells lines, compounds, concentrations, time, *etc.*):

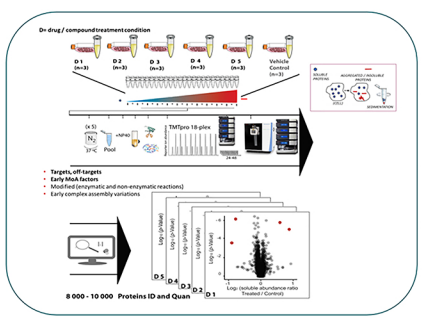
1. **PISA assay[1] + HOLSER[2]**
2. **Phospho Proteomics**
3. **PISA-Express + PTM Proteomics + HOLSER**
4. **PISA-Express**
5. **RedOx Proteomics + PISA-Express (PISA-REX)**
6. **Expression Proteomics**
7. **Expression Proteomics (e.g. FITExP or ProTargetMiner) + HOLSER**



Deep and proteome-wide chemical proteomics approaches to identify direct targets and MoA

[1] Proteome Integral Solubility Alteration (PISA) assay:
identification and quantification of **protein solubility changes**, high throughput and high multiplexing

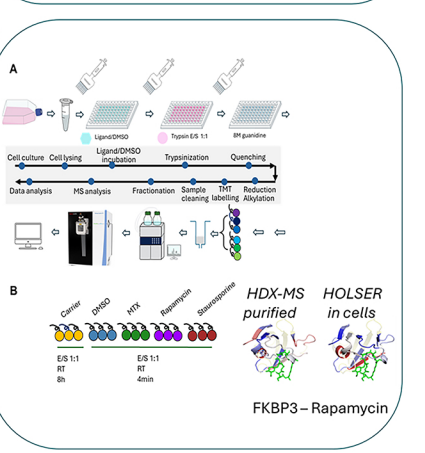
(2018-2019, First publication: doi:10.1021/acs.jproteome.9b00500)



[2] HigH-ratiO partiaL proteolysiS with carriER proteome (HOLSER):
a proteome-wide **structural protein accessibility** profiling assay; high protein sequence coverage and high multiplexing

- *Structure Profiling and Site-resolved Ligand-Protein Interactions;*
- *Orthogonal to PISA for Target deconvolution and MoA;*
- *Complementary to HDX-MS*

(2026, First publication: doi:10.1021/acs.analchem.6c04688)



24

A data-independent acquisition method with high-resolution precursor selection improves protein identification and quantitation for high on-column loadings of complex digests

Knudsen Anders [3]

[3] SCIEX

Data-independent acquisition is a widely used technique for the deep quantitative analysis of complex proteomics samples. Methodologies to improve coverage while achieving the highest quantitative precision and accuracy are essential for the application of DIA workflows towards disease research. ZT Scan DIA is a method utilizing a continuously scanning quadrupole for precursor isolation, and has been demonstrated to improve both qualitative and quantitative performance relative to discrete-window DIA methods. This work demonstrates how increased precursor selection resolution through the use of narrower Q1 isolation windows, thereby reducing chimeric MS/MS spectra, improves protein identification and quantitation, particularly with higher on-column loadings of complex lysates. K562 lysate tryptic digest was diluted in buffer and analyzed at 50ng and 250ng on-column loadings. Nanoflow reverse-phase chromatography (32-minute active gradient) was performed on a Waters M-Class HPLC system using an IonOpticks Aurora XS Ultimate column. Analysis was carried out on a ZenoTOF 8600 system (SCIEX), using either an 85 window (variable-width) Zeno SWATH DIA method, or ZTScanDIA methods with either 5Da or 2Da Q1 precursor isolation window widths. DIA data were processed using both DIA-NN software version 1.9.1 and PEAKS Studio version 13.1, using a K562/HeLa spectral library. The results show that with 2Da Q1 precursor isolation windows, protein group identification improved by up to 8.5% compared to Zeno SWATH DIA, and that quantifiable protein groups and precursors increased by 11.5% and 21.4%, respectively. Spectral quality is also significantly enhanced using narrower precursor isolation windows due to the ability to select precursors with higher resolution.

Transforming quantitative sensitivity for small molecule analysis using a high-resolution workflow

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[1] SCIEX

Quantitation of small molecule therapeutics in complex biological matrices requires methods that meet proper selectivity and sensitivity needs. Quantitative analyses of small molecules have commonly been performed on a gold-standard triple quadrupole mass spectrometer. However, more recently, such analyses have been increasingly conducted on a high-resolution mass spectrometer given the improved selectivity, which, consequently, aids in enhancing sensitivity. In this study, quantitation of small molecule therapeutics in extracted rat plasma was performed on a novel QTOF mass spectrometer.

In this study, 6 small molecules were analyzed in an extracted rat plasma matrix. Each calibration point was analyzed in triplicate. On average, an 18-fold improvement in LLOQ was achieved across 6 small molecules compared to a previous generation QTOF mass spectrometer using a high-resolution MRM mode. Representative small molecules such as alprazolam, diazepam, and trimethoprim reached an LDR of up to 4.5 orders of magnitude, demonstrating measurement across a wide range of concentrations. Overall, LLOQ accuracy was between 80% to 120% and accuracy for all non-zero calibrators was between 85% and 115%. The overall %CV was <13% for all small molecules analyzed.

Since the analysis was performed on a high-resolution-mass-spectrometer, compounds showed improved selectivity compared to a traditional triple quadrupole mass spectrometer analysis. In the case of diazepam, a 6.5-fold improvement in signal-to-noise (S/N) was achieved when compared to an MRM analysis on a triple quadrupole mass spectrometer. This was attributed to the capabilities of a high-resolution mass spectrometer, which enabled the differentiation of the target small molecule from matrix components.

Enhanced metabolite identification and quantitation using continuously scanning Q1 data-independent acquisition

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[1] SCIEX

Data-independent acquisition workflows are increasingly applied in small molecule metabolomics because they enable simultaneous qualitative and quantitative measurements. However, conventional fixed window DIA suffers from limited precursor specificity due to co isolation of multiple ions, leading to complex fragment spectra and reduced confidence in metabolite annotation. Here, we evaluate a Zenotrap enabled scanning DIA workflow that introduces a continuously scanning Q1 isolation window, providing an additional precursor specific dimension to DIA data. We further assess a narrow window implementation designed to enhance specificity for isomeric and closely related metabolites.

NIST SRM 1950 human plasma was protein-precipitated, and the supernatants were analyzed by high-resolution mass spectrometry coupled to an ExionHPLC system. Chromatographic separation was performed using a Waters ACQUITY Premier CSH Phenyl Hexyl column (2.1 × 100mm, 1.7µm) with a 19-minute gradient of 0.1% formic acid in water and methanol. Data were acquired using data dependent acquisition (DDA), standard ZT Scan DIA. The continuously scanning Q1 isolation was combined with Zenotrap pulsing to maximize MS/MS duty cycle and fragmentation sensitivity. Performance was evaluated based on precursor specificity, spectral complexity, annotation confidence, and metabolome coverage.

ZT Scan DIA improved precursor to fragment ion assignment compared with DDA and conventional fixed window DIA by leveraging the continuously scanned Q1 dimension to facilitate data deconvolution. This resulted in reduced spectral complexity and increased confidence in metabolite annotations. In NIST 1950 plasma, ZT Scan DIA enabled improved differentiation of isobaric and isomeric metabolites and minimized precursor co-isolation without compromising MS/MS data quality or metabolome coverage.