id #89604

The application of DIA-MS coupled with PISA TPP for drug-target deconvolution

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Thermal proteome profiling (TPP) enables unbiased identification of protein drug targets and potential off-target effects. The technique relies on monitoring the ligand-induced thermal stability changes across the proteome in a protein's native state. Cellular Thermal Shift Assay (CETSA)[1] and Proteome Integral Solubility Alteration (PISA)[2] are two leading MS-based target engagement studies utilising the TPP principle. CETSA-MS measures the shift in the protein's melting curve, based on ligand-induced solubility changes. In a similar approach, PISA-MS measures the difference in the integral protein abundances, without the curve fitting required in CETSA. PISA overcomes the ineffectiveness of CETSA in characterising proteins whose thermal profile does not fit into a sigmoidal melting curve. To overcome the cost of isobaric labels, a requirement of a high-resolution instrument to deconvolute isobaric masses, and the need for complex data analysis for curve fitting during CETSA, we adopted a label-free approach to analyse PISA pools by data-independent acquisition (PISA-DIA). The lysate PISA-DIA analysis workflow was tested on four overlapping temperature gradients containing eight temperatures each (37 °C – 49 °C, 44 °C – 56 °C, 51 °C – 63 °C, and 58 °C – 70 °C). This is the first PISA-MS study of its kind known to use overlapping gradients as a means of increasing the resolution by providing additional data points to establish the precise drug target under investigation. For this purpose, human multiple myeloma cells expressing BCL-xL (BCL2L1) were lysed and either treated with an established BCL-xL inhibitor (Venetoclax, 1 µM and 2 µM) or DMSO in quadruplicates. PISA pools were analysed by diaPASEF on a timsTOF Pro instrument (30 min gradient/sample) following USP3 digestion[3] and StageTip peptide cleanup. The PISA-DIA analysis identified the known drug target (BCL-xL) in both drug concentrations within the 44 °C – 56 °C gradient. This benchmarking study confirms the robustness, suitability and cost-effectiveness of the current PISA-DIA workflow for routine target engagement studies.

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id #89683

Mass spectrometry imaging of brain signalling systems reveals abnormal alterations induced by parkinsonism and L-Dopa therapy

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Neurotransmitters and neuropeptides are important signalling molecules in the brain and alterations in their expression levels have been linked to neurological disorders such as Parkinson's disease (PD). Here we used ultrahigh mass resolution Fourier-transform ion cyclotron resonance (FTICR) MALDI-MSI for the comprehensive mapping of neurotransmitter networks and neuropeptides in specific brain regions. Our new reactive MALDI matrix (FMP-10) facilitated the covalent charge-tagging of neurotransmitters and metabolites containing phenolic hydroxyl and/or primary or secondary amine groups, including dopaminergic and serotonergic neurotransmitters and their associated metabolites. We illustrate the capabilities of the developed method on brain samples from an experimental PD model (MPTP), including L-Dopa-induced dyskinesia (LID) in PD.

We imaged the metabolism of L-Dopa and the catecholaminergic pathway in brains from LID and non-dyskinetic animals, chronically treated with L-Dopa. L-Dopa and the L-Dopa metabolite 3-O-methyldopa were abnormally highly elevated in the whole brain of dyskinetic animals resulting in significant increases in dopamine and downstream metabolites in all brain regions, except putamen and caudate. Dopamine formation was correlated with serotonin in specific layers of hippocampus and cortex in LID but not in the putamen. Furthermore, we found that dyskinesia severity correlated with the levels of some abnormally processed peptides, notably, destyrosine dynorphins, substance P (1-7), and substance P (1-9) in multiple brain regions. Our results demonstrate that the abundance of selected active neuropeptides is associated with L-DOPA concentrations in the putamen, emphasizing their sensitivity to L-DOPA. Additionally, levels of truncated neuropeptides (which generally exhibit reduced or altered receptor affinity) correlate with dyskinesia severity, particularly for peptides associated with the direct pathway (i.e., dynorphins and tachykinins). The increases in tone of the tachykinin, enkephalin, and dynorphin neuropeptides in LID result in abnormal processing of neuropeptides with different biological activity and may constitute a functional compensatory mechanism for balancing the increased L-DOPA levels across the whole basal ganglia. This study

highlights the capacity of MALDI-MSI to elucidate the dynamics of neurotransmitter and neuropeptide signalling during PD and its treatment.

id #89645

Determining the antigen processing requirements for spliced peptide presentation

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The HLA-I peptide (pHLA-I) pathway leads to the presentation of peptides derived from endogenous antigens (including cancer-associated antigens) to T-cells for immunosurveillance. Collectively, these cell surface-presented peptides make up the cell's immunopeptidome. Although they make good candidates for CD8^{+} T-cell immunotherapy, clinical trials with peptide vaccines have shown poor overall response rates. This creates an urgent need to expand the repertoire of novel peptide antigen targets. To broaden this repertoire, we used a previously published bioinformatic tool (Hybrid Finder) to analyse the immunopeptidome and detect spliced peptides. Spliced peptides are formed by a transpeptidation reaction occurring in the proteasome, and studies have shown that they could represent up to 30% of the immunopeptidome. They are an attractive inclusion in peptide-based vaccines as they broaden the repertoire of available targets and studies have shown that these peptides are immunogenic in nature. Whilst peptide splicing has been attributed to proteasomal-catalysed processes, it is unknown what effect other components of the antigen processing and presentation machinery (APPM) play. In this study, we used a combination of a panel of APPM knockout cells as well as inhibitors of various components of the APPM. Some initial immunopeptidomics analyses of a melanoma cell line treated in the presence or absence of an inhibitor of ERAP, an APPM component, showed that there was no significant change in the proportion and abundance of spliced peptides presented between different treatments. Since there was no abrogation in spliced peptide presentation, this suggests that other components could impact spliced peptide presentation. We anticipate that the knockout of TAP or components of the proteasomal complex would abrogate spliced peptide presentation thus conclusively explaining splicing.

id #89464

Multiplexed single cell proteomics for investigating cellular heterogeneity during hypoxia

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Cellular diversity is a ubiquitous property of biological systems and is exemplified in tumour cell heterogeneity. Investigating cellular heterogeneity requires single cell resolution in order to identify population subtypes or temporal phenomena, such as cellular differentiation. Single cell resolution is afforded by well-established sc-RNASeq methods, where transcript abundance is frequently taken as a proxy for protein abundance. Realistically, protein abundance is the integral of mRNA translation rate which limits the application of mRNA as a proxy of protein abundance to constitutively expressed genes. Methods for high throughput single cell proteomics using LC-MS have gained recent traction with developments in peptide multiplexing reagents and high resolution Orbitrap instruments.

Here, we present an implementation of a multiplexed single cell proteomics (scMS) workflow to semi-quantitatively investigate the diversity of oxygen-deprived HEK293 cells which are cultured in bioreactors. From this data, we were able identify hypoxia-driven heterogeneity in single cell proteomes. Meaningful protein profiles can be drawn from this data which corroborate literature findings from bulk-sampling methods. Here, we outline our implementation of an scMS workflow and discuss conclusions drawn from its utility in investigating hypoxia-driven stress in industrially relevant HEK293 cells.

id #89674

Identification of oxidatively modified proteins to utilise as biomarkers of immune cell activation

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Inflammation is evident in chronic diseases and condition, and the inflammatory response involves the pathogenic generation of oxidants including hydrogen peroxide and hypochlorous acid. Oxidants can also oxidatively modify the side chains of proteins, and we propose that the measurement of such in biofluids such as blood would be useful as reporters of inflammation and therefore severity of many diseases. One such disease, is the fatal childhood muscle wasting disease Duchenne muscular dystrophy (DMD), which currently has no effective treatment. The exact pathological mechanisms of DMD are not understood, however we and others have proposed that white blood cells, particularly neutrophils, contribute to the pathology. To explore the potential of oxidatively modified proteins to be readouts of disease pathology in biofluids, we used immunoassays and mass spectrometry to identify oxidatively modified proteins in the blood of mice DMD model. A modification of particular interest is the halogenation of protein tyrosines because this modification occurs only when a tyrosine residue encounters hypochlorous acid, which is specifically produced by the enzyme myeloperoxidase, an enzyme produced in neutrophils. We identified a protein in plasma that is 26% more halogenated in dystrophic animals compared to wildtype (n=7-8). We show that the halogenation of this protein (hal-X) correlates strongly with muscle pathology (including inflammation). To further investigate the use of halogenation of this protein as a readout of disease pathology, we measured hal-X in plasma from dystrophic mice treated with

taurine, an anti-inflammatory drug we have previously shown to be effective at decreasing inflammation and muscle cell death [1]. We show that in the mouse model, a decrease of 15% in halogenation of this protein occurs after taurine treatment (n=8), which again correlates with other readouts of muscle pathology. Our results provide evidence that oxidatively modified proteins, particularly halogenation, have the potential to be useful biomarkers of disease pathology in conditions associated with excess inflammation, and these can be readily detected in blood. These biomarkers could be utilized for monitoring putative treatments in DMD, where currently the testing of potential therapies is limited by clinical outcome measures that require 12-18 months to properly determine efficacy. We hypothesize that the measurement of oxidatively modified proteins in biofluids could report the efficacy of prospective treatments within a few weeks, which would significantly reduce the cost and time burden of clinical trials in DMD and numerous other conditions.

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id #89668

An Untargeted Method for the Detection of Post Translational Modifications

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Introduction: The term proteoform is used to describe the base sequence of a protein plus or minus any chemical modifications that the protein might have. This term is given after post translational modifications (PTM's) occur on the base sequence. Detection of PTM's is typically done with enrichment kits or by using open or predictive search algorithms to see which post-translational modifications are present within the peptides of a proteoform. The problem that is faced with these approaches is that when multiple proteins are obtained from a sample, digested, and cleaved into peptides we can't know which protein from a sample had which modifications that we detect.

The focus of this work was to investigate a method to detect modified peptides in the context of their parent proteins without requiring an enrichment kit, by separating proteins based on their isoelectric point. Our hypothesis that this method should allow for the determination of the context of modified peptides

Method: Mouse lungs were obtained from surplus tissue from a previous larger study. Protein extraction and solubilisation was carried out using a novel in house method followed by isoelectric focusing. Focused IPG strips were transferred to a gel and run using 1D SDS page. Then stained with Coomassie Brilliant Blue. For this pilot study the top row of each gel was used and cut into 12 equal size pieces. Proteins were digested using an In Gel Digestion Protocol. Samples were stagetipped and then prepared for analysis using a mass spectrometer.

Results: The densitometry report results showed that the extraction method was successful. Overall 2500 proteoforms were identified with >20 different modifications found including acetylation of lysine and phosphorylation, from just a single strip from the top of a 2D gel This new method greatly reduces the number of proteins that are analysed per spot to ~20. Which provides greater chances that the modified peptides are within the 12-15 dominant ions that are picked up by mass spectrometry analysis using a Top N method. In addition, this method allows the modifications to be detected alongside their respective parent protein. Hence the method allows for post-translational modifications to be detected simultaneously. Further study is currently being analysed to look at the PTM's present in two separate whole gels (192 total samples).

id #89384

Quantitative targeted and global proteomics reveal novel biomarkers of disease in mouse models of a rare-genetic epilepsy disorder

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SCN2A encodes a voltage-gated sodium channel and *de novo* variants are the cause of autism and a spectrum of developmental and epileptic encephalopathies (DEEs). In this study, mass spectrometry is utilised to measure the brain proteome to assess the role of altered protein expression in various phenotypes of SCN2A DEE as well as identify potential novel biomarkers for the disorder. Brain samples were collected from WT Bl6 mice and mice heterozygous for SCN2A R854Q and S1759R mutations at postnatal (P) day 25. Samples were digested with trypsin/LysC and desalted using C18 cartridges and analysed on a Shimadzu 8050 LCMS Triple Quadrupole *(QQQ)* mass spectrometer for targeted quantification of SCN2A protein, as well as on a Thermofisher Orbitrap Eclipse for global proteomics. An experimental spectral library was generated from representative pooled samples from our experiment. Targeted quantitation of SCN2A protein showed no significant changes between models and brain regions. The consensus spectral library created for the DIA experiments contained 6,576 unique protein IDs and 47,917 unique peptides. An average of 4757 shared proteins were quantified using DIA between WT and SCN2A mutant models, with an average of 210 and 621 significantly up and down-regulated proteins, respectively. SCN2A protein is not differentially expressed suggesting dysfunction in neuronal firing previously seen is due to functional effects of the mutation. Proteins up and down regulated in the SCN2A models are associated with pathways related to neuronal firing, metabolism, synaptic plasticity and behaviour, suggesting these proteome changes could influence the wide range of symptoms seen in patients with SCN2A DEE.

id #89621

A novel platform integrating single cell muscle physiology with single cell proteomics

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It is well known that exercise is key to reducing risk factors associated with disease and conferring positive health benefits in addition to promoting optimal skeletal muscle function. Understanding how exercise-regulated signalling mediates the beneficial effects of exercise may lead to improved therapeutic strategies and in this context, we recently identified the uncharacterised protein C18ORF25 as a novel regulator of skeletal muscle function and validated it as a bona-fide AMP-activated protein kinase [1]. We further showed that loss of C18ORF25 results in reduced muscle contractile function at the whole muscle and single muscle cell level.

Here, we present a newly developed proteomics platform that allows functional assessment of excitation-contraction (E-C) coupling pathways in an individual muscle cell to be integrated with single cell proteomics in the exact same cell. This platform has been used to identify specific defects in sarcoplasmic reticulum calcium loading following loss of C18ORF25, with paired single-cell proteomics identifying Calcium/Calmodulin Dependent Protein Kinase II Gamma (CAMK2G) as down-regulated and thus a likely key driving mechanism of the reduced muscle function observed in the absence of C18ORF25. Furthermore, our platform has associated seven discrete phenotypes of the E-C pathway to >1,600 proteins using correlation analysis resulting in the identification of 851 unique phenotype:protein associations. Our data analysis workflow shows hundreds of novel associations with an example of two positive controls being the correlation between (i) Junctional Sarcoplasmic Reticulum Protein 1 (JSRP1) and (ii) Sarcoplasmic Reticulum Histidine-Rich Calcium-Binding Protein (HRC) and calcium handling phenotypes.

We believe our unique proteomics platform, integrating single-cell physiology with single-cell proteomics in the same cell, will provide deep mechanistic insights into skeletal muscle dysfunction in diseased states like insulin resistance and type-2 diabetes not possible with other research strategies. In addition, the ability to pinpoint defective proteins in diseased conditions will allow for novel therapeutic interventions to improve skeletal muscle contractile and metabolic function.

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id #89367

How do Greater wax moth larvae survive on a plastic diet?

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Plastic waste pollution poses a global ecological challenge[1]. Plastic breakdown through biological systems such as insects could be a future solution for plastic waste management. The larvae of the Greater Wax Moth, *Galleria mellonella* (Gm), and/or their resident gut microbiota have been shown to degrade plastics, including polyethene (PE)[2-4] and polystyrene (PS)[4]. The degree of biodegradation and actual carbon metabolism from plastic is an active area of research. The biochemical adaptations the larvae recruit to survive on plastic waste remain unknown. Herein, we fed Gm larvae six types of plastics for 7 days and dissected the larvae to separate body and gut samples for Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS)-based proteome measurement. We found that the larvae responded to each plastic type differently, and detected bioactive peptides associated with the larval nutrient stress management mechanisms. Notably, larvae fed with expanded low-density PE foam possessed markedly altered metabolic responses with significant changes in enzyme categories such as hydrolases and oxidoreductases. Network analysis revealed that the plastic diets impacted the Gm body proteome; specifically, proteins involved in triglyceride breakdown, lipid metabolism, energy storage and production markedly increased within the PS-fed larvae, while muscle-building proteins were increased in the PE fibre and PS-fed larvae. These results demonstrate the biochemical shifts incurred given dietary plastic supplementation and provide a shortlist of enzymes from the insect and gut microbial community for follow-up and assessment for use at the industrial scale.

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id #89042

Painting a Picture of the Gynaecological Cancer *N*-Glycome and Proteome

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Gynaecological cancers are a group of tumours that originate from the vulva, vagina, cervix, uterus (endometrium), placenta, fallopian tubes and ovaries in adult women. These cancers are known to undergo epithelial-mesenchymal transition (EMT) which results in the metastasis of epithelial cells and an increase in their resistance to apoptosis by altering the extracellular matrix (ECM). ECM proteins play an imperative role in cell health as they provide the scaffold upon which cells and tissues are built. Hence ECM proteins directly and indirectly influence almost all cellular processes, including cell differentiation, proliferation, and motility. The majority of ECM proteins are known to be heavily glycosylated, most commonly *N*-glycosylated whereby glycans (i.e., complex sugars) are attached to asparagine residues. Previously, our group has employed MALDI mass spectrometry imaging (MSI) using axial TOF instruments to spatially map *N*-glycans across formalin-fixed paraffin-embedded (FFPE) ovarian and endometrial cancer tissue sections (1,2). In this study, we have further investigated the *N*-glycome and proteome of FFPE ovarian, endometrial, cervical and vulvar cancer tissue sections by MALDI-MSI using Bruker's latest timsTOF fleX instrument. Furthermore, our group has established an *in-situ* tandem mass spectrometry (MS/MS) fragmentation protocol which can be implemented post-MALDI-MSI analysis to structurally characterise and confirm cancer-specific *N*-glycans and tryptic peptides. Lastly, our group has successfully separated structural isomers (i.e., *N*-glycoforms) *in-situ* using trapped ion mobility mass spectrometry (TIMS). With these complimentary methods in mind, a wealth of information can be acquired from a single tissue section, thereby reducing the sample preparation time previously required while increasing the number of *N*-glycans and tryptic peptides identified relative to conventional axial TOF instruments. It is with this information that we can better understand glycan and protein alterations within gynaecological cancers in the pursuit of discovering diagnostic or therapeutic targets.

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id #89421

Development of a lectin bead-based diagnostic test for oesophageal cancer

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The diagnosis of oesophageal adenocarcinoma, or Barrett's oesophagus , a pre-malignant condition associated with an increased risk of the adenocarcinoma, currently requires expensive and invasive endoscopy-biopsy procedures. These are often only performed when obvious symptoms have manifested, typically at a late stage disease progression. A targeted mass spectrometry-based assay was developed to analyse serum samples for oesophageal adenocarcinoma and Barrett's oesophagus. The assay was based around a magnetic bead bound lectin that pulls down glycoproteins with specific sugar moieties before digestion and LCMS analysis. The method was automated and optimised to measure 33 target peptides in the lectin pulldown in a short 20 minute mass spectrometry run. Analysis of an initial cohort (n=50) showed the method to be robust and reproducible with an average intraday CV of 9.3% across the 33 peptides and an average interday CV of 11.5%. The panel of serum protein biomarkers that were measured correlated with the presence of early stage oesophageal adenocarcinoma (high grade dysplasia). A larger cohort (n=266) was analysed and used to build statistical models which distinguished between disease status, using protein biomarker measurements and simple clinical parameters. Several developed models achieved good discrimination with AUROC values ranging from 0.89-0.97. Validation of the models was undertaken in the smaller cohort, with the two best performing models achieving AUROC values of 0.82 and 0.87. This proteomics based assay has the potential to produce a clinically viable diagnostic test to support screening and early detection in populations at high risk of oesophageal adenocarcinoma and Barrett's oesophagus.

id #89423

Development of a diagnostic test for defining obstructive airway diseases

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Labelling specific obstructive airway diseases is a difficult task as traditional clinical entities of asthma, chronic obstructive pulmonary (COPD) and bronchitis have overlapping clinical features and symptoms. Currently diagnosis of obstructive airway diseases is always based on lung function tests that are not always definitive to diagnose the overlapping conditions which can result in inappropriate treatment. The early identification and differentiation of phenotypes of obstructive airway diseases would be of benefit for targeted treatment. A previous experiment had identified a set of potential biomarkers from a discovery proteomics workflow and this current work details the targeted mass spectrometry assay that has been developed and applied to a large cohort of 350 individuals from the Busselton Health Study. The patient samples were stratified according to different clinical variables in a general population. The data from the patient samples was then compared across the individuals and to the original discovery experiment to determine significant biomarkers for each definition of the obstructive airway disease. These

biomarkers have potential as a simple diagnostic tool to assist in the early diagnosis and precise treatment for obstructive airway diseases.

A proteomics based predictive assay for diabetic retinopathy

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Diabetic retinopathy occurs when the retina is damaged due to complications from diabetes. It affects a third of type 2 diabetics and is a leading cause of vision loss around the world. Current clinical tests can diagnose diabetic retinopathy once the eye is already damaged, but the tests are subjective and delayed, and cannot predict future development of disease. The aim of this project is to discover and develop protein biomarkers in plasma for early diagnosis of diabetic retinopathy that allows earlier intervention and prevention of future progression of disease.

Participants in the Fremantle Diabetes Study (FDS) were assessed for diabetic retinopathy through a traditional eye scan that grades the severity of disease. These patient plasma samples were then analysed by a traditional proteomics workflow to identify biomarkers of retinopathy. A 48 patient cohort was selectively pooled into the following four distinct groupings:

- Group 1 = Diabetics with no retinopathy at baseline and did not develop retinopathy at year 4
- Group 2 = Diabetics with no retinopathy at baseline but developed retinopathy at year 4
- Group 3 = Diabetics with mild/moderate retinopathy at baseline and year 4
- Group 4 = Diabetics with severe retinopathy at baseline and year 4

A triplicate iTRAQ 2D-LCMS experiment was performed for initial candidate biomarker selection with a total of 31 potential biomarkers identified (20 prognostic-only, 3 diagnostic-only and 8 prognostic plus diagnostic candidates). A targeted LCMS assay was designed to measure the level of these biomarkers and applied to a new cohort of individual samples (n=49). The biomarkers developed from this study have potential in a clinical assay for prediction of diabetic retinopathy.

Postmortem interval estimation using an untargeted LC-MS/MS-based proteomics approach

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Introduction/Background:

Accurate estimation of time since death and the postmortem interval (PMI) are crucial in forensic medico-legal investigations to understand case circumstances (e.g. narrowing down list of missing persons or in-/exclude suspects). Current methods, however, heavily depend on the experience of the forensic examiner such as subjective assessment of morphological changes, stiffness and discolouration. The aim of the current study was to utilize an untargeted LC-MS/MS-based proteomics workflow to study time-dependent postmortem changes of peptides/proteins in human muscle tissue to help establish more objective biomarkers for PMI estimation.

Methods:

Minimal-invasive thigh muscle tissue biopsy samples were collected at the Australian Facility for Taphonomic Experimental Research (Sydney) from 9 donors across multiple time-points after death (≤120 days postmortem). Samples were homogenised and proteins extracted. After reduction (TCEP) and alkylation (IAA), protein extracts were cleaned up (SP3) and proteolytically digested (trypsin). Samples (n=179) were analysed in triplicate on a nanoHPLC system coupled to a SynaptXS ion mobility TOF-MS (DIA mode, scan range 50-1500 m/z). Progenesis Qi for Proteomics was used for data processing and peptide/protein identification (reviewed human database). Sample collection time-points were converted to accumulated degree days (ADD) utilizing daily average temperatures (ºC). Using R, all possible ratios between peptides originating from the same protein were calculated, log2 transformed, filtered according to linear regression results (r^2 and slope) and database matches manually confirmed.

Results:

A total of 11 promising peptide ratios were found for potential PMI estimation up to ADD = 655 (≤48 days, n=6 donors). These ratios displayed consistent and robust linear relationships (increasing/decreasing) over time across all donors, originating from myosin-7 and actin (alpha skeletal muscle) proteins. Similarly, 4 promising ratios of peptides from myosin-2 protein were found for PMI estimation up to ADD = 1535 (≤120 days; n=4 donors). Linear, season-specific peptide ratios were found for bodies decomposing in spring/summer (n=4 donors; 3 peptide ratios from myosin-7 protein) but not for bodies decomposing in autumn/winter (n=5 donors).

Discussion/Conclusions:

All peptide ratios with significant linear regressions were calculated from peptides (non-modified and with oxidation posttranslational modifications) that originate from muscle tissue-specific proteins (myosin and actin) and hence are likely related to the *in-vivo* decomposition of muscle tissue. While identifying peptide ratios with consistent and robust postmortem ADD-dependent changes is a first step towards objective PMI estimation, increasing the number of donors is necessary to conduct an extensive modelling approach for prediction of PMI for unknown forensic case samples.

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id #88926

DIA-based in silico spectral reference libraries, an improved alternative for peptide quantification in clinical samples via DIA-NN

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Data Independent Acquisition (DIA)-MS data is traditionally analysed using spectral reference libraries (SRLs) created from Data Dependent Acquisition (DDA) experiments. DDA inherent missingness, low reproducibility and biased to high abundant peptides, have allowed the surge of alternative strategies such as SRL-free methods and gas phase fractionation (GPF). In this study, we compare the performance of several SRL strategies in the context of complex clinical samples using DIA-NN.

1,261 fresh frozen cancer samples encompassing 73 cancer types were grouped to produce 39 sample sets. Each set was fractionated using high-pH RP-HPLC, fractions were analysed in Triple TOF 6600 instruments in technical duplicate, DDA and DIA (fractionated DDA and fractionated DIA), respectively. Additionally, every set went through GPF and resulting fractions were acquired in DIA (GPF DIA). Lastly, *in silico* SRLs were produced per set from unfractionated sample DIA files (SRL free DIA). The four approaches were evaluated in terms of library size, quantitated peptides, q-value scoring, missingness and peptide intensity.

In a prostate adenocarcinoma set, fractionated DDA, fractionated DIA, SRL-free DIA, and GPF DIA, approaches identified 5,087, 7,876, 6,974, and 6,927, proteins, respectively with a peptide overlap of 32%. Whilst fractionated DIA had the highest number of identifications, the SRL-free DIA method had the lowest number of missing values at 38%. Analysis of the proteins uniquely identified in each show that the SRL-free method produced the most intense values with the highest confidence score peptides and the lowest missingness.

Conventional DDA-SRLs performed poorly relative to the other methods in terms of uniquely quantified peptides and proteins and peptide confidence score. Fractionated SRLs also have a cost of a high proportion of missing values and low intensity peptides. Our results show that the SRL-free approach offers a higher quality alternative for large scale clinical proteomic analysis where sample availability may be limited.

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id #89201

The characterisation of protein binding from in vivo paediatric ECMO circuits

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Background:

Extracorporeal Membrane Oxygenation (ECMO) is an artificial heart-lung machine for critically ill patients with severe respiratory or cardiac failure (1). Despite providing critical support, there is an increased risk of bleeding and clotting due to the continuous contact between blood and circuit (2). Whilst previous studies have extensively investigated blood samples from patients on ECMO (3, 4), protein and cellular adsorption to ECMO circuit, as a factor that could potentially contribute to clinical complications, has largely been overlooked.

Aims:

To characterise the proteins bound to ECMO circuits collected from children on ECMO.

Methods:

This study was approved by the Royal Children's Hospital ethics committee (HREC/15/RCHM/123). ECMO circuits were collected when removed from patients. For each circuit, samples from 5 different sites were collected (Figure 1). Protein quantification and characterisation was performed using bicinchoninic acid protein assay and Data-independent acquisition Mass Spectrometry (DIA-MS), respectively. Identified proteins were processed using the Reactome Over-representation Pathway Analyses tool (5) to determine corresponding functional pathways.
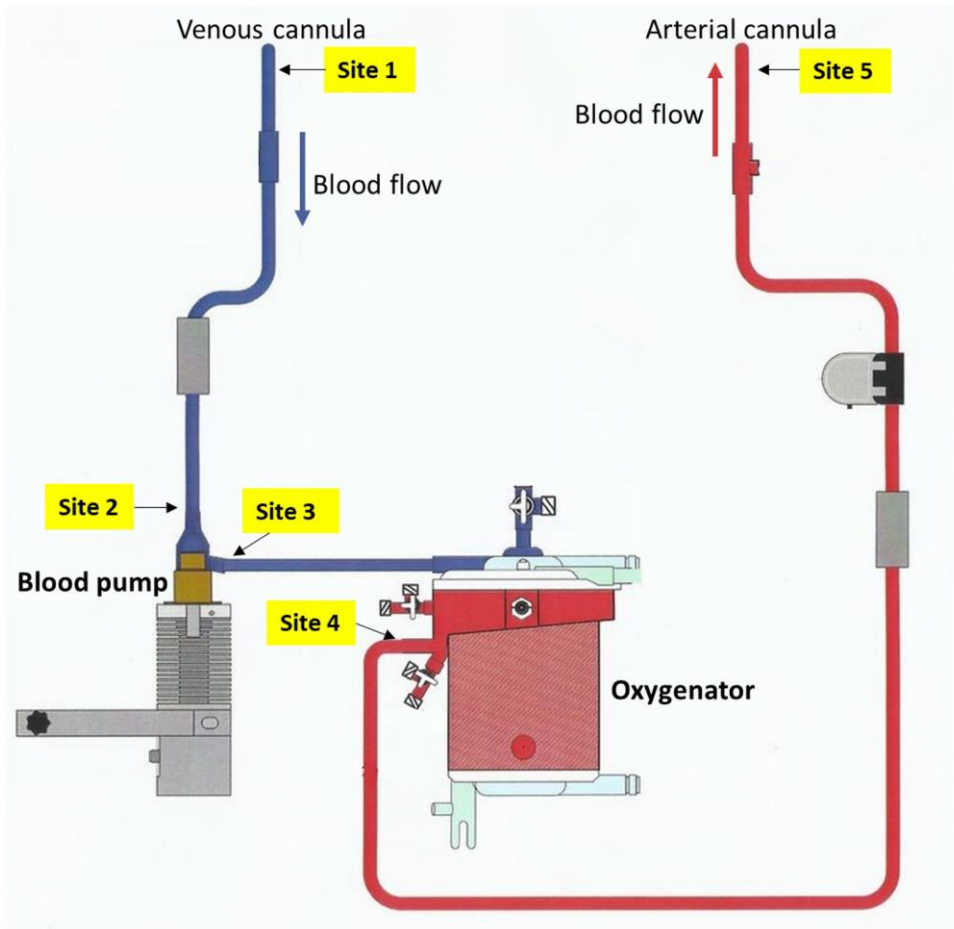

Figure 1. The basic diagram of ECMO circuit. Circuit samples were collected from Site 1: Post venous can 2: Pre-pump head; Site 3: Post-pump head to oxygenator; Site 4: Post-oxygenator; Site 5: Pre arterial cannu

Results:

Seven ECMO circuits were collected from six paediatric patients, with Patient 1 requiring a second run of ECMO support (Named as Patient 1.1 and 1.2).

The median protein concentration of all samples (n=35) was 16.3ug/mL (Range: 0~76.9ug/mL). In site-matched patient comparisons, the protein concentrations of Patient 4 were significantly higher than that of Patient 5, while in patient-matched site comparisons, site 1 samples were significantly higher than site 2, 3 and 5 samples.

Samples with at least 20ug protein (n=19) were analysed by DIA-MS. The number of proteins identified within each sample varied, with a median of 2138 (Range: 913~3073). Specifically, albumin, haemoglobin, apolipoprotein E, apolipoprotein A-I, fibrinogen, and band 3 anion transport protein were the most abundant proteins identified in most samples. Hierarchical clustering and principal component analysis showed samples from the same ECMO circuit clustered together (Patient 1.1, 1.2, 3 and 6), except in Patient 4 where samples showed significant differences between sites (Figure 2). The pathway analysis demonstrated that identified proteins were enriched in pathways related to cell cycle, protein metabolism, coagulation, and inflammation.
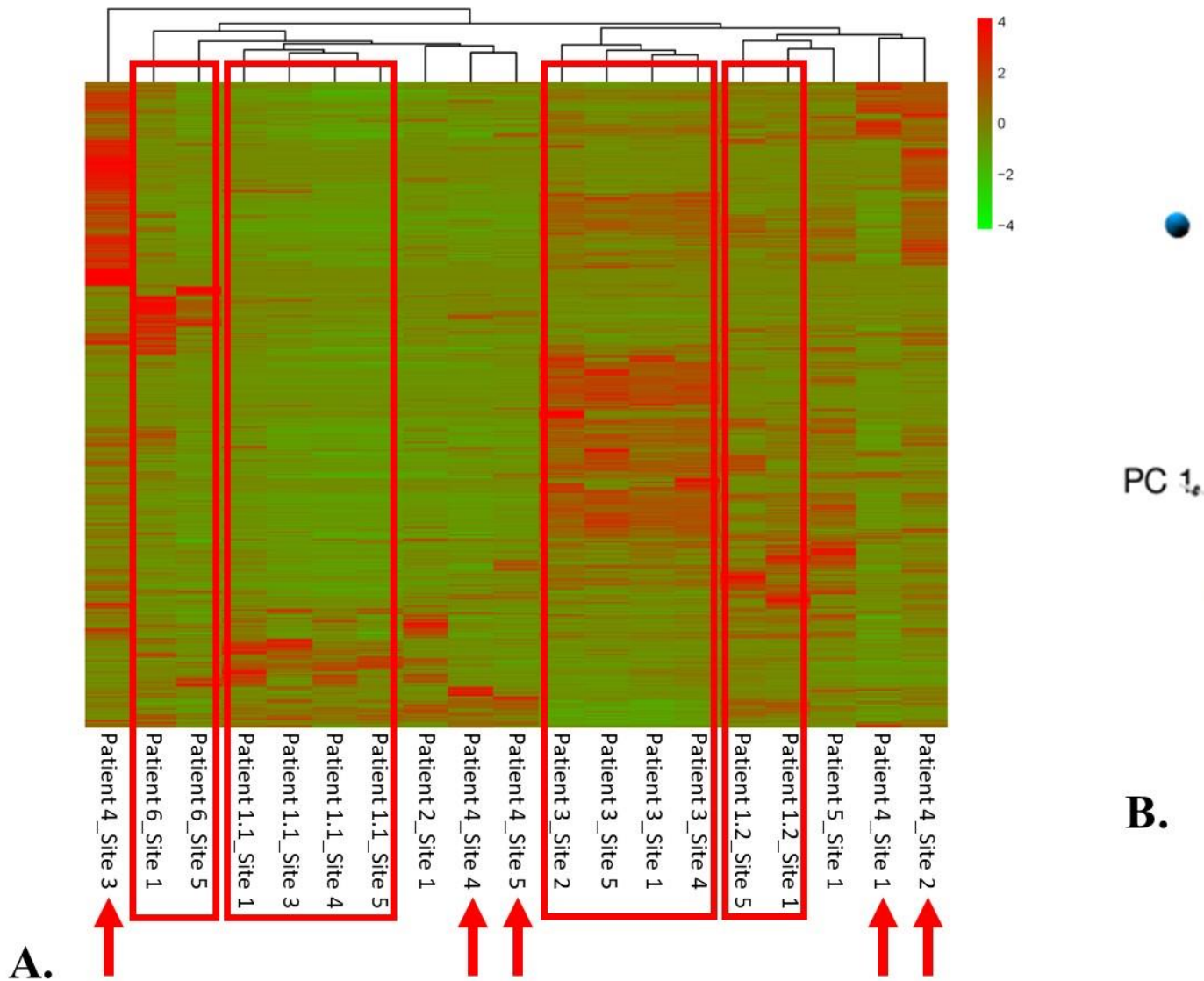
Figure 2. The unsupervised hierarchical clustering (A) and Principal component analysis (B) of circuit sam... of samples from the same patients of Patient 1.1, 1.2, 3 and 6, red arrows show separate sample grouping...

Conclusions:

This is the first study to characterise the proteins bound to ECMO circuits collected from paediatric patients utilising proteomic approaches. Composition of bound proteins was heterogeneous between different patients and between different sites. The bound proteins were enriched in pathways of coagulation and inflammation, which may be associated with clinical outcomes and provide a potential area for further interventions.

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Identification of the Doublecortin like kinase 1 (DCLK1) interactome reveals novel kinase dependent processes involved in gastric cancer progression.

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Introduction: Gastric cancer (GC) is one of the most aggressive and 3rd in cancer related deaths worldwide. Since the cancer genome atlas molecularly profiled GCs many new promising targets have emerged for Eppstein-Barr virus positive, microsatellite-, and chromosomal instable GCs. Unfortunately, no new therapeutic targets have emerged for the genomic stable (GS) subtype, which has the lowest overall survival. However, Ser/Thr-protein kinase Doublecortin like kinase 1 (DCLK1) is significantly upregulated in GS GCs. DCLK1 is a microtubule associated protein family member, hence important for cellular shape, polarity, migration, mitosis, and vesicular transport. DCLK1 has been shown to promote epithelial-to-mesenchymal transition and inducing migration and invasion in many different solid cancers. Therefore, we aimed to investigate whether DCLK1 could be a potential therapeutic target for GS GCs and determine how molecularly DCLK1 contributes to gastric cancer progression and identifying kinase substrates.

Methods/Results: DCLK1 overexpression resulted in increased cellular protrusions and cell migration *in vitro,* which was reversed upon kinase inhibition with the highly specific DCLK1-IN-1 inhibitor. Subsequent label-free quantitative (phospho)proteomics revealed significant changes in RNA-processing, cell-cell adhesion, cell cycle processes, cellular matrix organization, chromatin organization, and vesicular transport upon either DCLK1 overexpression or inhibition. 91 DCLK1 interactors were confidently identified with the Significance Analysis of INTeractome (SAINT) software on the quantitative affinity purification coupled to mass spectrometry (AP-MS) experiment on FLAG-tagged DCLK1, DCLK1-IN-1 treated, and kinase dead mutant. Next we compared the interactome and phospho-proteome data and identified 22 overlapping potential DCLK1 kinase substrates. These 22 proteins are again involved in membrane trafficking, RNA pol II transcription, RNA processing, ribosome biogenesis, cytoskeletal-, mitotic spindle-, and chromosome organization.

Conclusion: Our comprehensive proteomics approach revealed novel reversible processes for DCLK1's contribution to cancer progression and identified potential kinase substrates. Together, this study establishes DCLK1 as a promising targetable regulator of GC.

Characterisation of peptide and protein post-translational modification by cyclic ion mobility

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Post-translational modifications of proteins are key regulatory events for cellular systems, where proteins are covalently modified through addition of functional groups, such as phosphoryl or glycosyl groups, or cleavage events. These modifications can alter the structure and function of proteins, which can affect the role of proteins in biological systems. PTMS can usually be detected easily by mass spectrometry due to a change in peptide mass based on the type of modification, though this does not always yield site specific information. The specificity of the type and position of the modification and produce very different effects on protein function, hence the ability to specifically identify PTMs sites is crucial to understanding how these modifications affect protein function.

Ion mobility enables the separation of isomeric peptides and proteins that may be otherwise indistinguishable by traditional LC-MS. Furthermore, the cyclic ion mobility instrument allows for unique MS^n experiments, where species can be separated by IMS, selected and re-injected for further IMS separation. This enables very selective experiments, speciation of PTMs and in-depth characterisation of proteins and peptides. Here, we demonstrate the capacity for cIMS to separate, characterise and identify a range of common PTMS, including phosphorylation and glycosylation, in both simple and complex mixtures. The ability to more clearly separate and characterise PTMs will enable greater insight into the role these moieties play in biological systems.

The WEHI Proteomics Facility: tools, techniques and technologies

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The WEHI Proteomics Facility is a major technology platform integrated within the Advanced Technology & Biology Division providing the latest mass spectrometric instrumentation to facilitate researcher-led exploration, identification and quantification

of proteins and peptides. Our team of six experts in mass spectrometry-based proteomics utilise advanced proteomic technologies to facilitate the seamless translation of results to our collaborators. From experimental design and sample preparation, to data analysis and statistics – we offer expertise through all stages of the proteomics workflow. We work collaboratively with both academic researchers both at WEHI and externally, and offer fee-for-service arrangements with commercial organisations.

Our mission is to provide access to state-of-the-art liquid chromatography mass spectrometry-based proteomic and protein characterisation services with rapid turnaround times. Our four core research interests are methods development, mass spectrometry analysis, statistics & data analysis, and clinical discovery & translation. We facilitate a variety of mass spectrometry-based proteomics experiments including: global proteomics (DIA and DDA), drug-target identification, N-terminomics, post-translational modification (PTM) analyses (including phosphophorylation and ubiquitination), targeted mass spectrometry (PRM/SRM), trotein/recombinant protein identification, intact 'top-down' proteomics, cell-surface labelling, TurboID/BioID, crosslinking mass spectrometry (XL-MS) and interactomics (e.g. Co-IP, HA-tagged, FLAG-tag)

Here we provide a detailed outline of our collaborative research services, high resolution instrumentation and highlight our recent achievements in terms of instrument utilisation and method developments.

id #89615

A role for protein lysine acetylation in bile resistance in *Campylobacter jejuni*

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Campylobacter jejuni is the leading cause of acute bacterial gastroenteritis in the developed world. The biochemical mechanism of pathogenesis however, remain poorly understood. Lysine acetylation (KAc) is a reversible post-translational modification that may alter target protein function. A combination of acetyl-lysine immunoprecipitation and two-dimensional liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) identified 5,569 acetylated lysines on 1,026 *C. jejuni* proteins (~63% of the predicted proteome). Functional enrichment confirmed acetylated proteins are involved in key metabolic and virulence-associated pathways. Label-based LC-MS/MS quantified the unmodified proteome and KAc sites associated with growth in sodium deoxycholate (DOC, a component of gut bile salts). A total of 1,445 proteins and 3,571 KAc peptides were quantified. Of these, 761 acetylated peptides from 403 proteins were altered in abundance following growth in DOC, the majority exhibiting an increase in KAc. Protein changes were involved in metabolic pathways and processes, suggesting a dynamic and regulatory role for KAc in bile resistance. As with other enteric bacteria, we confirmed that KAc was largely chemically mediated by acetyl-phosphate (AcP), rather than *via* protein acetyltransferases, and this process involves the acetogenesis / acetate utilisation pathway and the sole bacterial deacetylase CobB that regulates acetyl-CoA synthetase (Acs). Unlike other bacteria, we noted several surface-associated virulence factors that displayed enrichment of multiple KAc sites. The major *C. jejuni* adhesin CadF was acetylated at 10 sites, several of which occurred proximal to known proteolytic cleavage sites that confer immune avoidance during infection. A reduction in KAc on these sites was shown to correlate with increased CadF processing, suggesting KAc may confer protection from proteases during periplasmic translocation and contribute to regulation of *in vivo* virulence. CobB inhibition did not attenuate global increases in DOC-associated KAc and had no influence on any acetylated sites in CadF. This work provides the first system-wide analysis of the lysine acetylome in *C. jejuni* and contributes to our understanding of KAc as an emerging modification in bacteria.

id #89583

Monitoring toxicokinetic properties of PFAS in rat models by targeted mass spectrometry

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Per- and poly fluoroalkyl substances (PFAS) are known as 'forever chemicals' because of their highly stable fluorinated carbon chains. These substances have been used in many applications such as fire-fighting foams, food contact materials, household products, cosmetics, and textiles due to their ability to withstand high temperatures and resist water, grease, and stains. However, it was found that PFAS have bioaccumulative, carcinogenic, and toxic properties negatively impacting human and environmental health systems. Human exposure can occur through many pathways including water, food and air resulting in PFAS in the blood. Although there are known health effects, examples include high cholesterol, pregnancy induced hypertension and cancers, the biological consequences of living with PFAS need to be further evaluated.

Here, we have established a method to detect PFAS from a rat model. Oral doses of PFAS, ranging from 5µg to 500 µg, were introduced into female Sprague Dawley rats through 20-16G metal gavage needles. The animals were housed in metabolic cages and samples of various matrices were collected at different timepoints over 120 hr. Proteins were removed from the samples containing PFAS using an organic extraction followed by purification of the supernatant using solid phase extraction (SPE). Samples were analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) by dynamic multiple reaction monitoring (dMRM) using an Agilent 6495B Triple Quadrupole Mass Spectrometer. We found that the various PFAS compounds had different accumulation, distribution, and elimination trends in rat models. These findings aid our understanding of PFAS toxicokinetic properties within biological systems.

Enhanced phosphoproteome coverage with gas phase fractionation combined with data independent acquisition (GPF-DIA)

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Data independent acquisition (DIA) methods have revolutionised the field of LC-MS based proteomics by increasing sample-throughput, enhanced data-completeness and, importantly, reducing the required sample input. However, truly comprehensive global proteomics remains difficult, due to the large dynamic range of protein abundances, differing by >6 orders of magnitude. Several methods have been developed to delve deeper into the proteome, including gas-phase fractionation (GPF) techniques (PAcIFIC, AIF, PulseDIA) (1-3) as well as SWATH-MS (4), which can dramatically increase the number of peptides and proteins that can be profiled through the generation of spectral libraries which has been shown to perform comparably to deep offline fractionation-based libraries for DIA data analysis. One advantage GPF has over older, offline fractionation-based techniques is that it requires less sample input. This is particularly useful for low abundant samples, such as *in vivo* cell populations. Here, we investigate how GPF-DDA and GPF-DIA techniques can be employed in tandem to profile the phosphoproteomes in these low abundant samples.

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Characterisation of branched ubiquitin architecture using Lb^pro and intact mass spectrometry

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Publish consent withheld

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Differentiation of lactyllysine and carboxyethyllysine modified peptides

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Post-translational modifications (PTMs) of proteins play a critical role in biological events by regulating activity, interactions, stability and degradation. The gold standard for identifying PTMs is high-resolution mass spectrometry, which relies on specific mass shifts caused by the modification, in relation to the parent peptide. Unfortunately, some PTMs can share identical mass shifts, making their differentiation difficult. Such PTMs include the recently discovered Lactyllysine (Kla) and Nε-carboxyethyllysine (CEL) which share the exact same mass shift of (72.02113031 Da). Kla is derived from S-lactoylglutathione or lactyl-CoA and CEL primarily from methylglyoxal (MGO). In recent years, several methods have been employed to confirm presence of Kla sites, including metabolic tracing using 13C6-glucose, however, all substrates responsible for Kla and CEL formation form as by-products of glycolysis. Therefore, the observed mass shift of 3.0101 Da in lactylated peptides would also be observed for those modified with CEL. Furthermore, several studies have used pan antibodies to generate an enriched lactylproteome. However, it was recently shown that these antibodies cross react with CEL modified proteins, suggesting the lactylproteome would also contain CEL modified peptides.

To investigate any differences occurring from Kla or CEL modifications, we generated peptides in-house by modifying BSA with S-lactoylglutathione and MGO, respectively. Initially, we found that each substrate preferentially modified different lysine residues, suggesting each modification may have different signalling consequences, further confirming the importance of their differentiation. Based on MS and MS/MS alone, there were no identifying features unique to either modification. Moreover, we also found that CEL modified peptides produced a cyclic immonium ion (156.103 Da), which was recently reported to be unique

to lactylated peptides. Interestingly, we found that Kla and CEL modified peptides have different retention times on C18 columns, with the former being more hydrophobic, eluting from the column ~6 min later than the latter when using a 105-minute gradient. Therefore, chromatographic separation of the two modified peptides may allow their differentiation.

Pancreatic Cancer HLA-peptidomics: An Unbiased Pursuit of Potential Targets for Immunotherapy

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Background: Despite the advances in overall cancer outcomes, pancreatic ductal adenocarcinoma (PDAC) remains among the deadliest cancers with a 5-year survival rate of less than 11%. As most cases of metastatic PDAC are resistant to conventional therapies, immunotherapy offers hope for improving PDAC outcomes and survival. To this end, an unbiased study of human leukocyte antigen (HLA)-bound peptides can facilitate future plans for T Cell-based immunotherapy. The goal of this study is to start with model cell lines and expand the pursuit to clinical specimens in the future. **Methods:** Panc-1 model cell line was cultured and treated with IFN-γ (50 units/ml) in a 72h time course with increments of 24h. Snap-frozen pellets of size 5e7 and 5e6 were collected for HLA-peptidomics and proteomics analysis, respectively. Induction of HLA class I molecules was assessed using flow cytometry as a readout for IFN-γ effects. HLA-peptidomics pellets were lysed and HLA-peptide complexes were isolated using the anti-pan-HLA class I antibody W6/32. Bound peptides were prepared for mass spectrometry according to established protocols. The proteomics pellets were lysed and processed based on the S-TRAP method. HLA-peptidome data was acquired on a Bruker TimsTOF Pro in a data-dependent mode and proteomics data was acquired on Sciex 7600 ZenoTOF in the ZenoSWATH mode. Datasets were searched with a 1% FDR at the peptide and protein level. **Results:** Panc-1 cells clearly show increased HLA class I expression upon IFN-γ treatment in a time-dependent fashion. Around ~25000 HLA-bound peptides in total were identified, with a time-dependent increase in peptide numbers post-IFN-γ treatment. A small subset of these peptides (0.4%) was considered cancer-specific; including known tumor-associated antigens (TAA) such as kinetochore protein Nuf5 and MAGEA1. A proportion of these peptides (0.11%) have been shown previously to act as immunogenic T Cell targets. Proteomics data shows a clear time dependence in proteome remodeling. Mainly, the members of the antigen processing pathway are upregulated earlier in the time course, while some proteins associated with cytoskeleton and metabolic remodeling are differentially expressed in the later time points. **Conclusion:** The established workflow is sensitive to identifying up to 25000 peptides from as little as 5e7 cells from a PDAC cell line that is usually known for low HLA expression. Additionally, detecting known T Cell epitopes and potential targets derived from TAA proteins shows potential for using the workflow on patient-derived material to detect peptides with potential for the possibility of (semi-)personalized immunotherapy based on biopsies.

Functional phosphoproteomic analysis of insulin signalling in ageing bone

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Insulin signalling in bone plays a critical role in development and the regulation of energy metabolism. However, a systems biology analysis to map *in vivo* signalling has yet to be performed. Furthermore, whether signalling is rewired during ageing and insulin-resistance is unknown. Here we present the first mouse bone phosphoproteome study of 8- and 73-week-old mice following acute *in vivo* insulin stimulation and identified >16,000 phosphorylation sites mapped to 4528 bone phosphoproteins, of which >4,600 sites are novel. Hundreds of phosphorylation sites were differentially regulated between young and old bone revealing dramatic rewiring and defects in insulin signalling. Kinase:substrate prediction using machine learning coupled to phosphosite evolutionary conservation analysis and integration with human bone mineral density GWAS enabled us to prioritise novel proteins containing differential phosphorylation and highly likely to play important roles in bone function. We next developed a CRISPR/Cas9 loss-of-function screening pipeline in zebrafish to assess the role of these proteins on bone development. This identified several novel insulin-regulated phosphoproteins as causal regulators of bone formation including AFF4, the core scaffold of the transcriptional Super Elongation Complex (SEC). Using targeted phosphoproteomics and affinity-purification coupled to mass spectrometry, we show that AFF4 is a novel substrate of nuclear-localised P70S6K, and phosphorylation regulates SEC formation. Furthermore, we show the activity of the SEC is defective under insulin-resistant conditions and is associated with reduced phosphorylation of AFF4 and an inability to engage efficient transcription of Immediate Early Genes. Taken together, we have defined *in vivo* defective insulin-signalling events in old mouse bone and identified novel functional phosphorylation events.

Mapping the druggable landscape of malaria parasite through thermal proteome profiling

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To achieve the ultimate goal of malaria elimination, a new generation of antimalarial therapeutics is required, that possess different mechanisms of action to drugs currently in clinical use. However, the identification of molecular targets and characterisation of mechanisms of action of antimalarial drug candidates to this day represents a critical bottleneck in the antimalarial drug development pipeline.

In order to provide novel avenues for the rational design of the next generation of antimalarial therapies, we carefully assembled a comprehensive library of over two dozens structurally diverse antimalarial drug candidates with different parasite-killing profiles and undetermined mechanisms of action. To identify their respective molecular targets and downstream cellular effectors of the drug-action, we used Cellular Thermal Shift Assay (MS-CETSA)[1,2]. MS-CETSA employs advanced quantitative mass spectrometry to interrogate the entire parasite proteome simultaneously and identify proteins thermally-stabilised under drug treatment, suggesting direct protein-ligand interaction. To maximise the information output, the MS-CETSA target engagement profiling was carried out for each drug in a multidimensional format including 10 compound concentrations, 4 thermal challenge temperatures and two sample types; assessing drug-protein interactions within intact *Plasmodium falciparum* infected red blood cells, as well as in the parasite lysate.

To further support MS-CETSA results, in parallel we carried out RNAseq-based drug-dose response transcriptional perturbation profiling for the assembled compound library, demonstrating highly specific transcriptional responses induced by different compounds. The molecular targets and drug mechanisms of action identified here provide novel directions for the development of future antimalarial intervention strategies and shed new light malaria parasite biology.

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id #89369

A multi-omics approach to characterising beer flavour

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The production and consumption of fermented beverages has been carried out for millennia by numerous cultures, and today represents a vast global industry of diverse products. In recent years, the beer brewing industry in particular has seen an explosion in the range of products with diverse sensory characteristics due to consumer demands for new beverages with diverse and interesting flavour and aroma profiles. Whilst made from relatively few and simple ingredients, the diversity of flavours and aromas found in beer can vary greatly, and much of this diversity is owed to the enzymes and metabolites produced by both the hops added during the brewing process and the yeasts used for fermentation. The increasing use of non-*Saccharomyces* yeasts in brewing, and the ever-growing variety of hops cultivars being used, requires a better understanding of the enzymatic and metabolic diversity of these essential components of the brewing process, to allow a more in-depth knowledge of their impact on the sensory qualities of the final product. Here we have taken a multi-omics approach using mass spectrometry-based metabolomics, lipidomics, and proteomics to characterize metabolic and enzymatic diversity in both non-*Saccharomyces* yeasts and Hops cultivars. We have identified significant differences in metabolite production between native wild yeasts and when compared to US05, a commercial *S. cerevisiae* brewing yeast.

id #89646

Unbiased plasma proteomics of a large type 1 diabetes cohort in a mass spectrometry facility – potential, perspectives and pitfalls

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The use of unbiased plasma proteomics remains a common first step for disease-based biomarker-discovery pipelines with the view that blood-based diagnostics continue to be widely implemented across medical settings. However, clinical translation from discovery-driven, proteomic surveys remains poor, with many published studies extensively studying few samples, and only a few studies attempting large-scale cohorts with greater potential for translation. While larger cohort sizes have arguable advantages, technical logistics and inherent variation often hold back the practicality and payoff of undertaking such studies in the clinical proteomics field.

WEHI Proteomics Facility recently undertook a large-scale plasma cohort from The Environmental Determinants of Islet Autoimmunity (ENDIA) Study. ENDIA is the largest global study to follow babies through pregnancy to explore factors which may protect against or impact upon the development of type 1 diabetes (TD1). The ENDIA cohort included 931 plasma

samples from mothers throughout pregnancy (Trimester 1-T3), as well as their infants from birth. This cohort was spread across 14 batches (96-well plates), totalling ~1200 samples with included controls and QCs (or ~2 months continuous instrument analysis time). Samples were manually processed using the USP3 method, and then acquired on a 30 minute analytical gradient (48 minute total cycle time) using diaPASEF acquisition on a timsTOF Pro, with two windows in each diaPASEF scan across 16 × 25 m/z precursor isolation windows (32 windows). Data was then searched on DIA-NN in library-free mode and the data subjected to in-house analysis pipelines for normalisation, imputation and protein quantitation.

Herein we discuss the logistics (both expected and realised) of running this large-scale ENDIA cohort through our proteomics facility. We detail aspects of our experimental design– from plate formats including sample, technical, batch and QC controls – as well as our sample processing and instrument pipelines, and how data was acquired, assessed, and analysed. We evaluate the success of this project from multiple perspectives – including as a facility – towards advising future proteomic analyses of large-scale clinical cohorts.

id #89316

Characterising the glycosylated immunopeptidome by leveraging diagnostic fragment ions using a novel spectral analysis program

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The immunopeptidome comprises the suite of human leukocyte antigen (HLA)-bound peptides that are presented at the cell surface for recognition by patrolling T cells. A variety of glycosylated peptides have been shown to be presented by HLA molecules, emphasising the importance of the antigen processing pathway in continuously monitoring intracellular protein states. However, little is known about the nature or extent to which glycosylated peptides represent a yet-to-be-explored component of the immunopeptidome, in part due to technical challenges for direct characterisation of sequence and glycan. To tackle these challenges, we have developed a program for the rapid analysis and characterisation of immunoglycopeptides in mass spectrometry data by harnessing the presence of signature oxonium ions. These methods include quantifying oxonium ion intensities to differentiate between N-linked and O-linked immunoglycopeptides, determining glycan makeup by comparing mass shifts between spectral peaks, and identifying the unmodified precursor peptide (Y0 ion) fragment in order to elucidate the composition of the attached glycan mass. By employing these techniques in tandem, immunoglycopeptides can be detected and characterised accurately and quickly, with analysis being completed in minutes on datasets containing tens to hundreds of thousands of spectra. This program provides not only a valuable method for rapid immunopeptidomic and proteomic interrogation but can also serve as a basis for more tailored downstream search strategies and analysis or to inform subsequent experimental design. These capabilities make it an ideal tool for exploring novel glycosylation events in the immunopeptidome, opening a new window into cell health and antigen presentation.

id #87880

MaxQuantAtlas produces an accurate large-scale concentration map of human E3 ligases and target proteins for next-generation TPD-based precision medicine

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Targeted protein degradation (TPD) has emerged as a promising new therapeutic modality. Heterobifunctional small molecule degraders degrade protein of interests by binding an E3 ubiquitin ligase and a target protein and hijacking the body's natural cellular degradation machinery. With over 600 known E3 ligases there are possibilities to utilize their diverse tissue expression profiles for selective TPD of targets in specific tissues, cell types and subcellular compartments for which clinical applications are limited because of unwanted on-target pharmacology. Therefore, an investment in data-driven accurate mapping of E3 expression across both healthy and diseased tissues is needed to identify E3s with tissue sparing potential.

With the widespread use of MS-based shotgun proteomics, countless datasets of different human cell types and tissues with deep proteome coverage are constantly being added to public repositories providing valuable quantitative information on proteome-wide protein copy numbers. However, it remains largely underused because of technical challenges to compare protein levels across individual studies.

Here we introduce MaxQuantAtlas, a quantitative proteomics software platform designed for the integration of MaxQuant-processed proteomics datasets over samples acquired with label-free and label-based quantification strategies and instrument types. Using PSM level data from individual MaxQuant-processed projects MaxQuantAtlas generates a unified protein-groups meta-table with newly assembled quantification results for all label-free, MS1-labeled, isobaric labeled DDA and DIA samples across all projects. For isobaric labeling samples, we introduce algorithms for ratio decompression and combined MS1-MS2 quantification to obtain cellular protein abundances. MS signals are matched to concentrations using the Proteomic Ruler method. An imputation method is used to make compatible the combining data from sources with different dynamic ranges. A two-dimensional quality score based on estimates of sample dynamic range and correlation of housekeeping proteins allows for detection of problematic samples leading to their automatic exclusion. Hence, we assemble a first-in-class human protein concentration atlas over cell lines, primary cells, healthy and diseased tissues which are clustering in biologically meaningful ways, independent of quantification and acquisition technologies.

The quantitative readouts of E3 expression from this software platform show good correlation with precision method results. Using comparative analyses of expression patterns from our protein concentration map, we are able to identify differentiated opportunities for selective pairings of E3 ligases with therapeutic targets of interest. We will provide examples of our LED (ligandability, expression, degradation) strategy in play as we select tissue sparing E3 ligases and showcase novel E3 ligase with broad utility for solid tumors.

id #89125

Elucidating a role for skin-derived filaggrin and keratin in *Schistosoma mansoni* cercariae infection

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Human schistosomiasis impacts over 250 million people worldwide and is caused by infection from *Schistosoma*, of which *Schistosoma mansoni* is among the most consequential species. The *Schistosoma* lifecycle involves the infection of a definitive mammalian host (including humans, rodents and non-human primates) by cercariae, a non-feeding infective stage which orientates towards mammalian host epidermal chemicals. Therefore, a promising and environmentally safe approach to minimise human schistosomiasis is to interfere with cercarial host identification mechanisms, which requires the identification of mammalian host-derived chemicals that induce behaviour change in the cercariae. This study demonstrated that unboiled mouse-conditioned water (MCW) induced a significantly greater excitatory response in cercariae (decreased passive behaviour) than boiled crude MCW and the control (Milli-Q water), suggesting the presence of behaviour altering proteins (BAPs). Following bioassay-guided analysis of RP-HPLC fractionated MCW, filaggrin and keratin were considered BAP candidates based on their presence across fractions that produced excitatory responses, their relative abundance within mammalian skin, and specificity to, and conservation within, mammals. Recombinant filaggrin induced similar excitatory responses to crude MCW and fresh mouse skin, while recombinant keratin induced increased cercarial directional change. In summary, this is the first report to elucidate the influence of host-derived proteins on *S. mansoni* cercarial behaviour, and more specifically, identify filaggrin and keratin as key inducers of behaviour change, which may inform the development of future *S. mansoni* biocontrols.

id #89586

Quantitative proteomics and phosphoproteomics analysis revealed the dual role of p120ctn as a tumor promoter and suppressor

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Neuroblastoma (NBL) is the most common extracranial solid tumor in children. Whilst, advances in treatment has significantly improved the survival rate of low-risk NBL patients, the overall survival rate of high-risk NBL group, especially N-Myc amplified patients, is poor. In this study, p120ctn was identified as one of the key players in regulating NBL aggressiveness. RNA-Seq and immunohistochemistry analysis on 206 NBL patients highlighted the mesenchymal-like signature of NBL tissue samples. It was revealed that high expression of p120ctn positively correlated with metastasis and poor outcome. Knockdown of p120ctn activated Wnt signaling and reduced the expression of the N-Myc. Depletion of p120ctn induced mesenchymal-to-epithelial transition which was highlighted by label-free quantitative proteomics and qPCR analysis of transcriptional factors. Moreover, contrary to the function of p120ctn as a tumour suppressor, p120ctn knockdown resulted in attenuation of angiogenesis, migration, invasion and sensitized the NBL cells to doxorubicin. The molecules that are regulating this opposing function of p120ctn was further investigated by phosphoproteomics analysis. The analyses revealed the differential activation of RhoA and Twist (S68) in a cell type-dependent manner. Targeting cell lineage with drugs in pre-clinical mouse models reduced the tumor burden and increased the survival of NBL bearing mice. Overall, this study highlights the dual role of p120ctn in cancer thereby highlighting a potential therapeutic avenue to treat NBL.

id #89694

Quantitative Proteomics for Understanding Histone Mutations in Human Disease

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Histones are small proteins that package DNA into chromosomes, and a large number of studies have showed that several post-translational modification (PTM) sites on the histones are associated with both gene activation and silencing. Along with DNA and small non-coding RNA, histone PTMs make up epigenetic mechanisms that control gene expression patterns outside of DNA sequence mutations. Dysregulation of these chromatin networks underlie several human diseases such as cancer. Here I will give an update on technology advancements that have allowed for high-throughput quantitative mas spectrometry analyses of histone PTMs and chromatin structure, and how we are applying these methods to understand epigenetic reprogramming found in malignant peripheral nerve sheath tumors (MPNSTs) and a neuroldevelopmental disorder. MPNST is an aggressive sarcoma with recurrent loss of function alterations in polycomb-repressive complex 2 (PRC2), a histone-

modifying complex involved in transcriptional silencing. Additionally, we have also helped discover novel mutations to the histone H3.3 gene that promote a new neurological disorder in children.

id #87953

Multi-OMICS of Gefitinib combined with tissue imaging to provide insight into potential hepatic toxicity

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An increasing number of tumours, including breast cancer, overexpress proteins of the epidermal growth factor receptor (EGFR) family. Gefitinib, a drug belonging to a class of tyrosine kinase inhibitors (TKIs), competes with ATP for its binding pocket in mutated or overexpressed EGFR receptors. This drug inhibits tyrosine kinase activity and thereby prevents cancer cell proliferation. Gefitinib is metabolised extensively in the liver by cytochrome P450 enzymes, primarily by CYP3A4 and to a lesser extent by CYP3A5 and CYP2D6. In this study, mice were administered the drug intravenously (10 mg/kg) and sacrificed at 0.5, 1, 3, 8 and 24 h post-dose (2 mice/time point). Extracts from liver tissues were then prepared (based on previously published methods) for LC-MS analysis, covering the proteome and lipidome. Additionally, tissue sections were taken for DESI MS imaging. Polar metabolite data were also collected for representative urine samples at the various time points. MS data for all extracts were collected using ion mobility enabled acquisitions for both LC-MS and DESI-based experiments. The resulting MS data were processed using a variety of informatic tools. Multivariate statistical analysis was performed for each of the OMIC studies independently prior to identifying the most statistically relevant features for pathway/network analysis. The results from the statistical analysis from these experiments showed similar clustering of the sample cohorts by time point (i.e., tracking that of the known pharmacokinetic trend) even with the exclusion of features related to the drug and its associated metabolites from the PCA and PLS-DA models, highlighting endogenous changes as a result of drug metabolism. Proteins associated with the p53 pathway, FGF and cytokine signaling showed expression changes. A number of bioactive phospholipids (PA's, PS's and PC's) were also identified as being significantly dysregulated following administration of the drug. Likewise, a variety of polar metabolites associated with a broad spectrum of pathways, including the tricarboxylic acid (TCA) cycle, glycolysis, lipid and amino acid metabolism. For example, tryptophan and taurocholic acid showed similar pharmacometabodynamic variation with that of the pharmacokinetic profile of the drug (i.e., increased abundance with a maximum at 3-8 h). Deoxyguanosine and asparaginyl-histidine on the other hand exhibited an opposite trend with down regulation over the same time points. The contrasting DESI imaging experiments of the livers also provided insight into the localisation of the drug/drug metabolites and endogenous species, which were identified from the LC-MS experiments at pre-dose, 0.5 and 8 h time points.

id #89301

Two cysteines are better than one - T cell recognition of peptides bearing penicillin-modified cysteinylated cysteine residues

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Penicillin is arguably one of the most successful antibiotics, however a subset of patients experience hypersensitivity reactions towards penicillin and other beta-lactam antibiotics, with drug-specific T cells implicated as mediators [1]. It is thought that penicillin facilitates the covalent modification of peptide antigens, resulting in the generation of novel haptenated epitopes that trigger drug-specific T cell responses [2]. As T cells are activated upon recognition of such peptides presented by human leukocyte antigens (HLAs), much effort has been placed into identifying penicillin-modified ligands that form the epitopes of penicillin-specific T cells. Upon identifying such ligands, we will be able to understand the interactions made between penicillin-specific T cells and the drug-modified HLA ligands. Due to the association noted between HLA-A*02:01 and the onset of drug-induced liver injury [3], we sought to understand how beta-lactam antibiotics might influence the HLA-A*02:01 immunopeptidome using the model drug benzylpenicillin (BP).

Lysine modification was previously reported as the primary site of covalent modification by penicillins, however we found large numbers of spectra containing BP diagnostic ions could not be explained by lysine haptenation. Instead, we observed that penicillin-haptenation occurred primarily on cysteine residues that had previously been modified by cysteinylation (76.4%). Synthetic versions of experimentally identified CysBP-haptenated peptides fragmented identically by CID (PCC 0.85-0.97), confirming sequence assignments and drug modifications. We further demonstrated that modification of the cysteinyl post-translational modification was employed by other beta-lactam antibiotics such as amoxicillin and flucloxacillin. Haptenation of cysteinylated cystines also occurred within proteins in lysates of antigen-presenting cells, providing a source of BP-modified

cysteinylated ligands for HLA presentation. Importantly, a patient-derived BP-specific T cell receptor recognised an epitope that was destroyed by reduction with TCEP, consistent with a BP-cysteinylated cysteine modification.

This is the first study to report drug-modified HLA-A*02:01 ligands. It highlights a novel target of penicillin (cysteinylated cysteines) which has been overlooked since haptenation was proposed in 1936 [4]. This will have major implications for deciphering the epitopes of T cells within the penicillin-induced immunopeptidome, and have the potential to be implemented in screening tools (e.g. peptide/HLA tetramers) to prevent the onset of the wider sphere of allergies induced by beta-lactam antibiotics.

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id #89053

IFNγ Modulates the Immunopeptidome of Triple Negative Breast Cancer Cells by Enhancing and Diversifying Classical and Non-Classical HLA Presentation

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To identify potential T-cell targets for Triple-Negative Breast Cancer (TNBC) vaccination, we examined the effect of the pro-inflammatory cytokine interferon-γ (IFNγ) on the transcriptome, proteome and immunopeptidome of the TNBC cell line MDA-MB-231. Using high resolution mass spectrometry, we identified in excess of 85,000 peptides from 9,647 source proteins presented by human leukocyte antigen (HLA)-I and HLA-II alleles. IFNγ resulted in increased number, diversity, and abundance of peptide antigens within the immunopeptidome, as well increasing the coverage of individual source antigens. Of note, these results were not mirrored intracellularly as we observed poor correlation of the immunopeptidomes with the cellular transcriptome and proteome. On deeper analysis of this multi-omics data, IFNγ showed remarkable induction of the antigen processing and presentation machinery including increased classical and non-classical HLA expression. In particular, HLA-E induction resulted in predicted presentation of cancer-associated and cancer specific antigens on the cell surface. Given the current lack of robust HLA-E-specific antibodies, we have pursued combined approaches to mitigate this and examine HLA-E bound peptides with greater fidelity and confidence. These findings will have significant implications on the immunotherapeutic and immunopeptidomic fields as the monomorphic nature of these molecules gives rise to potential pan cancer therapies. Furthermore, the ability of these molecules to present peptides other than leader sequences in perturbed states will fundamentally alter the way we view tumour evasion and escape.

id #89637

Investigating the structure-activity relationship of concentrated ionic liquids for their capacity to dissolve proteins for proteomic analysis.

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Present technologies for proteomics typically require analytes be in the liquid phase. Some analytes such as fibrous proteins, membrane proteins, and protein aggregates are intrinsically insoluble or have greatly decreased solubility in aqueous systems. This is often circumvented by surfactants, chaotropes, pH control, solid phase proteolysis (on-pellet digestion), and more.[1] However, recent literature has demonstrated the capabilities of a new class of chemical system, called ionic liquids, to dissolve intractable biomaterials containing insoluble biopolymers such as cellulose, and keratin.[2] Ionic liquids (ILs) are salts with low melting points, typically composed of an organic unsymmetrical cation (such as N,N'-dialkylimidazolium) and various anions such as chloride, acetate, tetrafluoroborate, etc.[3] Previous research has been centred around the dissolution of protein or polysaccharide material for purposes other than analysis, such as bio-material preparation, and bio-catalysis.[4] The use of ILs in bioanalytical chemistry of proteins has lagged behind, and of the few published investigations, most employ ILs in dilute aqueous solutions, where ILs are employed as cationic surfactants.[5] There is evidence to suggest that concentrated or neat ILs have unique properties and therefore may allow researchers to dissolve previously insoluble protein analytes. This has the potential to increase depth and robustness of sample preparation, and facilitate the analysis of intractable samples. Previous research using concentrated ILs for this purpose is sparse and there is a need to systematically investigate the structure-activity relationship between an IL's cation and anion structure, and its capacity to solubilise proteins. As an initial foray into the efficacy of these solvents for proteomics, bovine serum albumin was dissolved in a series of synthesised ionic liquids and aliquots were removed over time to monitor the dissolution progression by light microscopy, and the maintenance of the proteins primary structure by SDS-PAGE. The results confirm that hydrophilic ILs showed the best solubilisation capacity and that higher temperatures (in a restricted sense) improved solubility of the protein. However, higher temperatures and longer reaction times caused a reduction in the apparent molecular weight of the protein. Some preliminary investigations into the nature of BSA's modified primary structure after ionic liquid treatment have been conducted using LC-MS/MS based proteomics, but require further optimisation to be conclusive. Hence, for the present, researchers should exercise caution when using ionic liquids for protein analysis, until the full scope and limitations are known, an aspect we are presently investigating.

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id #89322

Assessing the impacts of Snomax, an ice-nucleating bacterium for creating artificial snow, on the Australian alpine environment

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Snomax is an ice nucleation inducer used to create artificial snow on ski resorts around the world, including some based in Kosciuszko National Park. The ice nucleation activity is reportedly derived from a protein purified from the fermentation of the *Pseudomonas syringae* bacterium. However, some strains of this bacterium carry numerous toxin and antimicrobial resistance genes. This project aims to accurately investigate the composition of this ice nucleation agent, and investigate whether it has caused any negative environmental impacts.

The first stage of the project involves detailed characterisation of the Snomax product. Amino acid compositional analysis demonstrated that it is approximately 50% protein, so further analysis of chemical composition is required in order to determine the other components. An initial proteomic characterisation using SDS-PAGE and nanoflow liquid chromatography – tandem mass spectrometry identified >1000 proteins, most of them related to *P. syringae* bacterium. Further proteomic characterisation will be performed in order to accurately detail the protein components involved.

The second stage of the project involves molecular analysis of field sampled soil and leaves from five different species of alpine plants. This was undertaken after the completion of the 2022 ski season, with samples collected at three different elevations from within the Thredbo ski area, and a matching set of three sites with similar geography located well outside the resort in the Dead Horse Gap area. The sites within the ski area had been exposed to the Snomax material for the previous four months, while those outside the ski area had not. Soil samples were collected, along with samples of leaves from *Eucalyptus niphophila, Nematolepis ovatifolia, Tasmannia xerophila, Ozothamnus secundiflorus,* and *Olearia phlogopappa*. The plant leaf samples will be analysed to determine whether the expressed proteome of plants exposed to Snomax is substantially different from plants collected from outside the resort area. The leaves will be analysed to determine if any significant differences in the phyllosphere (leaf microbiome) can be detected between sampling sites, and the rhizosphere bacterial communities from soil samples will be similarly analysed. The protein extraction and mass spectrometry analysis of the sampled leaves are underway, as are the DNA extraction and quantification of bacteria present in the phyllosphere and rhizosphere. Results from all these analyses will be presented in this study.

id #89685

Quantitation of the Iron Regulatory Hormone Hepcidin in Mammals

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The only input to total iron in mammalian systems, other than by clinical intervention is through diet. Because there exists no mechanism of controlled iron removal from mammals, iron regulation by physiological and biochemical recycling mechanisms, finely balanced by controlled dietary intake, each mediated by the peptide hormone hepcidin-25 (herein hepcidin) are integral to maintaining the strict requirements of iron homeostasis. With the physiological significance of this hormone well established, it has emerged as an informative biomarker. The use of laboratory mice as models of iron metabolism has greatly advanced our understanding of hepcidin and iron regulatory processes more broadly. It's recognised that an availability of animal models with a more representative iron turnover to that of humans would also be advantageous for human translation. More directly, hepcidin may yet prove useful for animal diagnostics. Here we reveal the first measurement of hepcidin from dogs by mass spectrometry and determine that previous reports of the amino acid sequence were contradictory to these data. Measurement of the peptide by mass spectrometry, following isolation from greyhound blood serum, revealed an amino acid sequence and peptide mass, differing from all accounts to date, yet demonstrating perfect sequence identity to that of the greater Canidae lineage of the Carnivora, as predicted by genome annotation. In the greyhound, the measured hepcidin peptide showed a similar temporal pattern to total serum iron, consistent with our understanding of hepcidin regulation of iron homeostasis, in agreement with expectations of human diagnostics, and providing translational evidence of the measured peptide being the iron regulatory hormone of the Canidae.

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Ensuring a suitable slice of the computational pie for Australian proteomics

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Traditional high performance computational infrastructure in Australia has not been designed for bioinformatics, and this presents challenges to many researchers in the life sciences, including proteomics. The Australian BioCommons has been engaging with members of the national proteomics community, including the Australasian Core Facilities group, since 2020 to understand the specific bioinformatics challenges facing researchers.

Challenges noted include a lack of sufficient compute (memory, cores), an imbalance between the software available for model and non-model organisms, the constant tension presented by the need for both open source and proprietary software, and the presence of high intellectual barriers that prevent the community from adopting new solutions to their challenges, whether these solutions are software, hardware or policy based.

In this presentation we will introduce the current scope of activities that the Australian BioCommons is coordinating in consultation with the Australian proteomics community that are aimed at addressing the challenges noted above. These activities range from development of discovery services for tools and workflows on local systems, deployment of tailored software and infrastructure that address proteomics requirements, establishing processes to allow information sharing with the broader community, and the beginnings of a collective national informatics training effort that supports the proteomics community. Specific examples include creating reusable/scalable DIA-NN workflows for use on a variety of computational infrastructures, and work with the freely accessible Galaxy Australia online bioinformatics workbench (https://usegalaxy.org.au/) to increase the size and complexity of possible MaxQuant analyses, make popular interactive tools like LFQanalyst available and expand the proteomics tool set available. Finally we are collectively exploring the use of the ARDC Nectar Cloud (https://ardc.edu.au/services/ardc-nectar-research-cloud/) for proteomics and development of a hosted proteomics-specific instance of Galaxy Australia.

We invite the Australian proteomics community to join us in the on-going effort to make sure that they have a fit-for-purpose and meaningful slice of the computational pie: the right tools, workflows and reference data, accessible on suitable compute systems, so that we can collectively empower both experts and end users within Australian proteomics.

If you would like to join the conversation, you are welcome at our new quarterly meeting series, where we discuss current community challenges and feature presentations from both local and international proteomics researchers. You can also join our Google Group: https://www.biocommons.org.au/proteomics

Systematic analysis of post-translational modifications in the yeast ribosome reveals extensive heterogeneity

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Protein translation is intricately controlled within the eukaryotic cell. While previously thought to be controlled exclusively through translation factors, it has recently emerged that the ribosome itself is important for translational regulation. Ribosome composition is highly varied within cells, a phenomenon called ribosome heterogeneity, and this is known to regulate selective mRNA translation. However, the contribution of protein post-translational modifications (PTMs) to ribosome heterogeneity remains poorly understood. Here we have combined ribosome profiling through Ribo-Mega SEC (size exclusive chromatography) [1,2] with mass spectrometry to systematically profile ribosomal PTMs in *Saccharomyces cerevisiae*. Ribo-Mega SEC allowed separation of distinct pools of ribosomes, from translationally active polysomes to unincorporated subunits, for downstream mass spectrometric analysis. Through use of multiple different proteases for protein digestion, we identified every yeast ribosomal protein (RP) except one, with peptides covering every single residue for the vast majority of RPs. This includes paralogous pairs of RPs that differ by only a few residues. We successfully identified and quantified all 12 known methylation sites on RPs, confirming that these are all present on actively translating ribosomes. Remarkably, half of these methylation sites were found to be substoichiometric on polysomes, indicating that these methylation sites contribute to ribosome heterogeneity. We also identified 18 phosphorylation sites across 14 ribosomal proteins. These were found at low

stoichiometry and on highly accessible, disordered regions of the ribosome, suggesting they may be actively regulated on intact ribosomes. Through open modification searches we identified several other PTMs on ribosomal proteins, including acetylation and ubiquitination. Lastly, quantification of paralogous RPs revealed that some pairs of paralogs are differentially incorporated into different populations of ribosomes. Together, our results reveal that PTMs contribute significantly to ribosome heterogeneity and provide a foundation for detailed studies on the roles of PTMs in translational regulation.

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3.

id #89202

Nutrition and allergenicity of food-grade protein extracts in novel food *Nannochloropsis oculata*

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Microalgae have great potential in becoming a sustainable source of dietary protein for human consumption. A marine microalgae — *Nannochloropsis* — has been commercially produced for its lipid content, but its high content of protein and essential amino acids (EAAs) provides an additional opportunity for food applications. To support the potential use of *Nannochloropsis oculata* in food products, the aim of this study was to evaluate a range of food-grade protein extraction methods and establish the nutritional quality and safety of the protein extracts.

Food grade (FG) protein extracts were obtained by hypotonic osmotic shock using milli-Q water. To identify the maximum number of proteins, a common proteomic extraction method (TCA-acetone followed by chloroform-methanol) was used in parallel. Food grade (FG) and non-food grade (NFG) extraction buffers were compared along with three different cell disruption methods including bead beating, probe sonication and a combination of both methods. The tryptic peptides resulting from both FG and NFG extracts were measured by data-independent acquisition (DIA) using a SCIEX 6600 LC-QqTOF and DIA-NN data processing. The data were used to identify and quantify proteins, including putative allergens using four different in-silico methods. Trace metals in the FG extracts were also measured to provide an overall understanding of the extracts' nutritional and anti-nutritional characteristics.

The effect of disruption methods on dried ground *Nannochloropsis oculata* was investigated in both FG and NFG extracts. Although the protein extracts obtained from all NFG methods had a similar number of identifiable proteins, there was a small improvement in identifiable proteins when bead beating (B) was used in food-grade protein extraction. Putative allergen protein assessment of the FG extracts identified five proteins with a high similarity to fish allergens. These include algal proteins with high similarity to fructose-bisphosphate aldolase in salmon and nucleoside diphosphate kinase in Atlantic cod. Metal ion analysis showed that FG protein extracts contained potassium, magnesium, and calcium, whereas chromium, arsenic and lead were at levels considered safe for human consumption.

In conclusion, for both FG and NFG protein extracts, applying cell processing methods beyond hypotonic osmotic shock on commercial ground *N. oculata* did not enhance protein yield; however, the identification of fish allergens deserves further investigation such as IgE binding tests.

id #89289

Towards the next generation in blood plasma analysis: improving analytical speed without compromising performance using improved databases, scanningSWATH and zenoSWATH.

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Plasma is the most studied human tissue due to its availability, potential for bio-marker discovery and central role in many illnesses including metabolic disease. Large scale human plasma studies have many unique challenges including large sample cohorts (>1000 samples) that may lead to issues with variability and batch effects. Secreted proteins undergo *in vivo* digestion events (maturation/processing/degradation) that can lead to proteins/peptides of interest not being detected. In our lab, we have begun to address these problems by creating a new FASTA database generation tool. This tool incorporates biological knowledge of in vivo digestion events and allows for specific detection of the "active" regions of secreted proteins. In addition, we have taken advantage of new mass spectrometry methods including zenoSWATH and scanningSWATH to reduce analytical time (<8min per run) without sacrificing sensitivity (>200 proteins quantified per sample) . Using our annotated database, we were able to significantly decrease search times compared to a semi-tryptic full database search, without causing a significant decrease in protein identifications of human plasma. This database could detect proteins that had been digested *in vivo* and was able to accurately quantify individual chains in a polyprotein such as insulin and IGF-1. Analysis using zenoSWATH allowed for the same detection sensitivity as scanningSWATH in 8 min but required 5 times less sample (2ug per injection). Together, these advancements in human plasma proteomic analysis have allowed for much faster analyses without reducing proteins identifications or sample reproducibility while also increasing our capability to detect secreted proteins and accurately quantify polyproteins.

Proteomic analysis of different varieties and species of rice under a range of abiotic stress conditions

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Rice is one of the most important food crops in the world, and the productivity of rice crops is threatened by a number of different environmental stresses. We have investigated the proteomic response of rice varieties and species with different genetic backgrounds, when exposed to a range of different abiotic stresses, including drought, high and low temperatures, and salt. This presentation integrates results from a number of different rice stress response studies performed in our laboratory over the years.

Physiological parameters including leaf water potential, photosynthetic and respiratory performance, and plant growth rates were measured. Proteins from tissues of young rice plants were extracted, peptides were separated using reversed phase nanoLC, and identified and quantified using high resolution orbitrap mass spectrometry, followed by peptide to spectrum matching.

In one study, plants from 8 different Oryza sativa varieties, and two other rice species, were subjected to drought stress and recovery. Proteins involved in proteolytic processing pathways were significantly increased in abundance, while many proteins significantly reduced in abundance in stress conditions were involved in photosynthesis. Some proteins were uniquely expressed in specific genotypes, while 8 proteins were up-regulated in response to drought stress in all genotypes, including actin-depolymerizing factor 3 (ADF-3) and GSH-dependent dehydroascorbate reductase 1. *O. australiensis* was able to retain more water in leaf cells, than the other two species, and a majority of proteins increased in abundance in stress conditions in *O. australiensis* were associated with photosynthesis and carbohydrate biosynthesis.

In a second study, rice plants were subject to multiple abiotic stress conditions simultaneously, with or without prior treatment with the stress hormone ABA. Leaf tissue from these plants was characterised at the proteomics and transcriptomics level. This allowed us to tease apart the tightly integrated networks of genes and proteins involved, highlighting the role of the TCA cycle and photosynthesis related proteins in complex networks acting in both stress response and ABA signalling.

Our studies on abiotic stress in rice over many years have identified a large number of novel stress response proteins. We have begun the process of functionally characterising some of these, by expressing them in yeast cells and screening those for stress response phenotypes. Initial data from that study will be presented here, which shows that homologues of certain uncharacterised genes are able to confer resistance to various stresses in yeast.

Non-small cell lung cancer biomarker discovery from un-fractionated blood cell pellets

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Blood biomarker studies are almost exclusively performed on plasma or serum. The blood collection and sample preparation steps are optimised to avoid haemolysis where possible because of the additional dynamic range issues arising from haemoglobin and the potential for artefactual results in immunoassays. In some clinical studies the cell pellet after plasma removal is stored frozen, although the lysis of cells exacerbates the sample preparation challenges for proteomics.

In this study we obtained plasma and frozen cell pellets from 16 patients with clinical stages II – IV non-small cell lung cancer (NSCLC) and 18 age and sex matched healthy controls. For proteomics analysis we have focused on the cell pellets and used a novel sample preparation technique to enable broad coverage of soluble, intracellular and membrane proteins. In a previous study we demonstrated detection and robust quantification from single-shot shotgun-LC-MS analysis of dried whole blood[1]. We used volumetric absorptive micro-sampling (VAMS) dried blood spot devices and loaded 30μL aliquots of thawed cell pellet sample. The samples were dried, washed, and trypsin digested *in situ*. Peptides were separated with a one hour gradient and quantified using DIA on a QE-HFX Orbitrap, producing ~3,700 protein IDs for each sample.

There were 508 differentially expressed proteins initially identified. Ingenuity pathway analysis revealed these identified proteins were involved with a variety of functional pathways covering adhesion and migration, as well as a strong network of known cancer-associated cytokines and enzymes.

To reduce complexity, proteins were ranked based on area under the curve (AUC), and separately using a boosted regression importance filter. The first filter produced a short list of markers that were both differentially expressed for the model and had an AUC > 0.9. The second filter calculated the importance rank of each protein using gradient boosting methods. The procedure was repeated 100 times, and the markers were ranked in terms of the number of times they were selected in the top 10 importance rank was recorded for each protein. Using the methods described above a set of 14 markers were identified using the AUC filter, and 13 markers were identified using the importance rank filter that discriminate between NSCLC and healthy controls with 3 markers common to both analyses.

Our rapid and reproducible methods enable the production of high-quality data from small aliquots of complex samples that are typically seen as requiring significant fractionation prior to proteomic analysis.

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Increasing the depth of single shot proteomics with enhanced data acquisition and processing strategies

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Advances in the online separation of complex proteomics samples including ultra-high performance liquid chromatography (UHPLC), ultra-high resolution separation columns, and high-field asymmetric waveform ion mobility spectrometry (FAIMS) enable a deeper mining of the proteome with single-shot methods. In addition to the improved separation of complex proteomes with single-shot methods described above, the CHIMERYS™ intelligent search algorithm unlocks the ability to deconvolute the chimeric spectra that still arise from the co-isolation and fragmentation of multiple peptides in tandem mass spectra from both Orbitrap and ion trap mass analyzers.

HeLa protein digests were resuspended in 5% ACN / 0.1% FA, and then injected via an autosampler onto analytical columns, and separated at 300 nL/min using a Thermo Scientific™ Vanquish™ Neo UHPLC system. Data were collected using a Thermo Scientific™ FAIMS Pro Duo™ interface and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer in data-dependent acquisition mode. Data files were processed with Thermo Scientific™ Proteome Discoverer™ 3.0 software.

To optimize the acquisition strategy for single-shot performance, 1 µg HeLa digest and a 1-hour gradient were used with isolation widths between 0.4 and 3 Th. Using an isolation width of 1.5 Th resulted in an average of 7,814 proteins, 59,213 peptides, and 128,647 peptide spectrum matches (PSMs) per run, increases of 13%, 31%, and 29% compared to a 0.4 Th isolation window, respectively. The use of a wider isolation window was enabled by the deconvolution capabilities of the CHIMERYS workflow, which provided 1.02 PSMs per spectra on average with the 1.5 Th isolation window versus 0.79 PSMs per spectra for the 0.4 Th isolation window. In contrast, processing the 1.5 Th isolation window results using a Sequest HT workflow provided an average of only 6,511 proteins, 39,549 peptides, and 53,289 PSMs. Thus, CHIMERYS provided improvements of 20% for proteins, 50% for peptides, and 141% for PSMs. To determine the impact of the increased isolation window on the ability to perform deeper analysis of longer gradient single-shot proteomes, 1 µg of HeLa were run using a 2-hour and 3-hour gradient. The 2 hour runs identified an average of 8,424 proteins, 76,619 peptides, and 187,391 PSMs, while the 3 hour runs identified 8,731 proteins, 84,084 peptides, and 248,076 PSMs. These results demonstrate that coupling advanced capabilities in online separation to enhanced data acquisition and intelligent data processing allows for substantial improvements in single-shot proteomics performance to yield a more thorough coverage of biological pathways with higher throughput.

id #88194

Utility of quantitative proteomics in solving rare disease diagnosis

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Diagnostic yields from exome (ES) or genome sequencing (GS) in suspected rare diseases are typically ~50% of the cases. Large numbers of candidate genes with variable genotype-phenotype correlations complicate variant curation and diagnosis, leading to some patients and families waiting for decades for a definitive diagnosis. Delayed diagnosis not only places a burden in public health systems but also prevents any possible patient intervention. Undiagnosed patients can have zero to tens of candidate variants that may warrant functional follow-up to determine genetic diagnosis. Label Free Quantitation (LFQ) and TMT-based proteomics were used to analyse primary fibroblasts from a retrospective cohort (N=10) of patients suspected of rare mitochondrial disease where ES/GS were inconclusive. We also comprehensively analysed (N=26) primary fibroblasts from diagnosed mitochondrial disease patients using LFQ Data Independent Acquisition (DIA) to validate and investigate the broad utility and limitations of the technique in the clinical setting. We achieved 80% diagnostic rate in the retrospective cohort with most variant types being intronic[1], missense and copy number[2] variants. We also showed that proteomics was more sensitive and specific in detecting protein defects compared to classical enzymology test, currently the only accredited test for confirmation of mitochondrial disease in Australia. Including the retrospective cohort, our quantitative proteomic approaches have helped achieve a genetic diagnosis for 30+ rare disease patients[3, 4], and contributed to the identification of 5 new disease genes that can allow faster diagnosis for future cases. Mass spectrometry-based quantitative proteomics has the potential to identify the functional impact of genetic variants by quantifying thousands of proteins in a single test, being a suitable approach for clinical accreditation and use in functional investigation of ES/GS inconclusive cases.

1.   1. Helman, G. et al. Multiomic analysis elucidates Complex I deficiency caused by a deep intronic variant in NDUFB10. Hum Mutat 42, 19-24 (2021).
2.   2. Frazier, A.E. et al. Fatal perinatal mitochondrial cardiac failure caused by recurrent de novo duplications in the ATAD3 locus. Med (N Y) 2, 49-73 (2021).
3.   3. Van Bergen, N.J. et al. Mutations in the exocyst component EXOC2 cause severe defects in human brain development. J Exp Med 217 (2020).
4.   4. Van Bergen, N.J. et al. Biallelic Variants in PYROXD2 Cause a Severe Infantile Metabolic Disorder Affecting Mitochondrial Function. Int J Mol Sci 23 (2022).

id #89266

Are You Ready for High-throughput Single Cell Proteomics with Great Depth of Coverage

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Low-flow liquid chromatography (LC) coupled to electrospray ionization-based mass spectrometry (MS) techniques have the capacity to probe extremely limited sample amounts. The increase in electrospray ionization (ESI) efficiency required is achieved by adopting narrow separation columns and reducing the LC flow rates to the "ultra-nano" (≤ 100nL/min) range.

Several aspects of ultra-low sample quantity analysis must be considered in the creation of robust and reproducible methods for this type of application. First, flow rate must be optimized for both sensitivity and throughput. Second, the LC platform must permit efficient sample analysis without wasting valuable MS acquisition time. Third, MS acquisition parameters must be optimized for the relatively low signal intensity observed from small sample quantities.

Here we describe a standardized LC separation setup together with 5 novel methods for balancing the sensitivity, throughput, and reproducibility required for routine analytics.

Experiments were performed on a Vanquish Neo UHPLC system coupled to an Orbitrap Exploris 480 mass-spectrometer. HeLa protein digest was separated on a 50 µm I.D. column at a flow rate of 100 nL/min. Contrasting data acquisition strategies, *i.e.*, data-dependent acquisition (DDA) and data-independent acquisition (DIA), were also compared for their impact on method performance.

Overall, we developed five ultra-low nano-flow LC-MS methods with gradients from 10 to 50 min, providing sample throughput of 24, 36, 40, 60, and 72 samples per 24 hours (up to 85% MS utilization). Using 250 pg diluted HeLa digest and the 10-min LC gradient (72 samples/day), ca. 800 protein groups were identified in DDA (Sequest), >1,800 protein groups in DDA (CHIMERYS), and ca. 2,100 – 2,700 protein groups in DIA (SN16 & DIA-NN18).

id #89268

Glycoprotein characterization combining native, PTCR and Direct Mass Technology mode mass spectrometry

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Introduction:

Glycoproteins are actively involved in physiological functions as well as certain diseases. However, glycoprotein characterization using native and native top-down mass spectrometry remains challenging as its heterogeneity leading to complex spectrum. In this study, we evaluated a few novel techniques including Direct Mass Technology mode and proton transfer charge reduction (PTCR) to unravel proteoforms of Human Fetuin A, a heavily glycosylated and phosphorylated protein, comprehensively under native condition. We further expand the application to heavily glycosylated Spike protein variants for determining their MW and oligomeric states.

Methods:

Human Fetuin A from human plasma was purchased from Sigma-Aldrich. Fetuin was buffer exchanged into ammonium acetate with Amicon 10K-MWCO or 30K-MWCO. Native MS and Direct Mass Technology mode were performed on Thermo Scientific™ Q Exactive™ UHMR. PTCR and EThcD analyses were performed on Thermo Scientific™ Orbitrap Eclipse™. Data were analyzed using Thermo Scientific™ BioPharma Finder™ 4.1 and STORIboard (Proteinaceous).

Results:

Initial native MS scan of Fetuin shows congested charge envelopes across m/z 3000- 4000. PTCR separated peaks which were previously overlapped in the full scan and thus increased number of identified proteoforms. Isotopically resolved proteoforms unambiguously discloses glycans and phosphorylations combinations. Native top-down analysis of Fetuin using EThcD proves the absence of signal peptide at N-terminus and demonstrates the B-chain is disulfide bonded to the N-terminal of A-chain through cys32-cys358 to form a loop. Top-down analyses of DTT released B-chain could yield 95% sequence and identify partially O-glycosylated site at Ser346. Direct Mass Technology mode analyses of Fetuin cleaned by 30K- and 10K-MWCO indicate different MW distributions with much more glycoforms detected compared to ensemble measurement. Such approach could also obtain MW, impurities, and trimer/monomer states of spike protein variants.

Conclusions:

Complementary mass spectrometry analyses including Native, PTCR, Direct Mass Technology mode, and EThcD could comprehensively characterize heavily glycosylated proteins.

id #89270

High Resolution DIA: A Workflow for Highly Accurate Relative Label-Free Quantification of Microbial Proteins in Complex Cell Lysates

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Introduction

Relative quantification of proteins in complex samples raises a demand for high sensitivity and reproducibility throughout large sample sets to gain meaningful insights on biological processes. Data-independent analysis has emerged as a powerful technique enabling quantification of thousands of proteins because it avoids the intensity bias and missing value problem that typically limit data-dependent methods. DIA in principle interrogates all peptides that are present in a sample and therefore is especially suitable for high-throughput and large-cohort studies.

Methods

Different microbial proteomes were spiked into a human proteome background at different ratios, yielding two proteome and three proteome mixtures with varying total protein amounts. Samples were separated on a 50 cm µPAC™ HPLC columns in direct injection setup on a Vanquish™ Neo system under nano-flow conditions. DIA experiments were run on an Orbitrap Exploris™ 240 mass spectrometer. Data was analyzed by Spectronaut™ 16 using a library-free approach.

Results

Using micropillar array-based column technology under nano-flow conditions for separation of peptides gives optimal peak shapes and intensities reproducibly over a long-term acquired data set with minimal performance loss. In connection with the high resolution DIA methodology, this enables for wide proteome coverage in two- and three-proteome mixtures as well as quantification accuracy below 10 % at high sample throughput. The uncomplicated and easily implemented library-free data analysis yields similar performance as low-key library-based approaches.

Conclusions

Micropillar array-based separation technology and high-resolution data-independent analysis enable for a wide proteome coverage at high throughput, while maintaining excellent quantification accuracy of relative protein ratios in complex cell lysates.

id #89271

Quantitative Multiplexed Analysis of Cancer Cell Lines Using Automated Sample Preparation with Increased Speed and Sensitivity

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Immortalized cell lines have played a key role in understanding the underlying biology underlying and as screening tools to identify drug targets in different signaling pathways related to diseases. Highly sensitive analytical method combined with standardized method for high quality samples preparation are essential in quantitative proteomics. Here we describe an end to end solution for quantitative proteomics analysis including automated sample preparation with customized reagent kit and Orbitrap Ascend using TMTpro™ workflow.

Sixteen different colon cancer cell lines were grown in four different batches and harvested in four different dates to assess the variability of a normal multiplexed experiment. $1x10^6$ cells from each cell line was harvested and rinsed with PBS. All the samples were processed using AccelerOme TMTpro™ kit was used together with the AccelerOme system. Peptides were analyzed on Orbitrap Ascend Tribrid Mass Spectrometer coupled to Vanquish™ Neo UHPLC system. Acquired data was analyzed using Proteome Discoverer™ software.

In quantitative analysis of large numbers of samples, quality of sample preparation, high confidence analysis with high throughput analysis are key aspects to address. AccelerOme system resulted in digestion efficiency above 92% and TMT labeling efficiency above 99% as the reaction chemistry while keeping artifactual modification below 1%. More importantly, sample preparation time were decreased from three days to six hands free hour, reducing the sample preparation time to 30% compared to a traditional manual method. The samples were analyzed using real time search (RTS) in Orbitrap Ascend Tribrid where architecture improvement has increased the ion management resulting in higher speed and sensitivity of peptide analysis and resulted in increase of 20% more peptides being quantified.

AccelerOme together with Ascend provides an ideal accurate and precise multiplexed proteomics workflow for mammalian cell line based proteomics research.

id #89613

Simultaneous targeted and discovery phosphoproteomics of cell signaling pathways using novel hybrid-DIA acquisition strategy

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Due to low stoichiometry of regulatory phosphorylation sites and high complexity of the phosphoproteome, achieving sufficient coverage by MS to cover all phosphorylation sites of interest is challenging. Targeted methods such SureQuant™ has proven useful in this context, allowing the precise quantification of dozens of targets even with limited sample amount. Here, we present a Hybrid-DIA method to extract precise quantitative information of selected phosphopeptide targets in parallel with profiling the global phosphoproteome in just one MS run per sample.

SureQuant™ Multipathway Phosphopeptide Standard (Thermo) combines 131 heavy-labeled tryptic phosphopeptides that correspond to phosphorylation sites of six relevant signaling pathways (EGFR, RAS-MAPK, P3IK/AKT/mTOR, AMPK, apoptosis and stress). Previously, using that mixture we were able to monitor phosphorylation changes in response to EGF and IGF-alpha in SY5Y cells. Here, we explored the sensitivity and detection limits of SureQuant™ method by using decreasing amounts (50, 25, 12 and 6 µg) of tryptic HeLa peptides for phospho-enrichment. Taking advantage of the higher sensitivity afforded by low-flow rate WHISPER gradients, we found that with 6 µg, SureQuant™ method provides quantitative information for 80 out of the 131 phosphorylation sites targeted.

Next, we benchmarked Hybrid-DIA against SureQuant™ to assess the potential to extract information from the full phosphoproteome in parallel with targeting the >130 phosphopeptides on the inclusion list. HeLa cells were grown in p6 dishes and treated 15 minutes with three kinase inhibitors: EGFRi (Lapatinib), MEKi (PD0325901) and P3Ki (Wortmannin), followed by stimulation with 100ng/ml of EGF for 10 and 90 minutes. The recovered peptides were mixed with 0.05 pmol of the Multipathway Phosphopeptide Standard mix and phosphopeptide-enriched with TiIMACHP beads. Samples were analyzed using either SureQuant™ or Hybrid-DIA using a 20 samples/day WHISPER gradient. Hybrid-DIA results recapitulated those obtained using SureQuant™ in terms of accuracy and sensitivity for the selected targets, whilst also providing information of ~4,000 phosphorylation sites from the global phosphoproteome. Importantly, the unbiased DIA analysis allowed us to identify ~200 phosphorylation sites differentially regulated by EGF and/or kinase inhibitors, revealing clusters of novel sites with specific kinase trends.

These results reveal the potential of the combination of targeted and discovery proteomics to maximize the information derived from phosphoproteomics experiments, which can be highly relevant in experimental contexts where samples with limited amount do not allow for multiple MS runs.

id #89323

High-throughput proteomics and phosphoproteomics of rat tissue using microflow Zeno SWATH.

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Microflow liquid chromatography-tandem mass spectrometry is a robust analytical tool for the high-throughput detection of peptides in clinical samples. In contrast, nanoflow is commonly used for the in-depth detection of phosphopeptides using long gradients and columns with smaller internal diameters. Here we explore a trap and elute micro-flow set-up for high-throughput proteomic and phosphoproteomic workflows using a Waters UHPLC system coupled to a ZenoTOF 7600 system.

For proteomic workflows, fresh frozen rat tissue from eight organs were analysed in technical replicates using 400 ng loads, a 30-minute gradient, a 5-minute trapping system, and a 50 variable window Zeno SWATH method. The proteomic data was processed using two computational pipelines to compare their performance. Protein identifications from rat brain, kidney, testis, lung, and spleen were similar across the two pipelines, however Fragpipe quantified significantly more proteins from liver (2700 vs 2500), heart (2300 vs 2100), and muscle (2100 vs 1700) than DIA-NN. Of the rat organs, kidney had the highest number of identifications with roughly 32000 precursors and 2900 proteins at 1% FDR using Fragpipe.

For phosphoproteomic workflows, 100 µg of rat tissue was enriched in phosphopeptides via titanium and zirconium IMAC magnetic beads, cleaned by micro-elution solid phase extraction and analysed in Zeno SWATH using a 15-minute or 30-minute gradient. The phosphoproteomic data was again processed using two computational pipelines to compare their performance. Spectronaut directDIA quantified significantly more class I phosphosites than Fragpipe (6608 vs 5764).

Both proteomic data from Fragpipe and phosphoproteomic data from Spectronaut showed distinct clustering of replicates for rat organs using principal component analysis. This indicates that both the proteome and phosphoproteome can distinguish between rat organs based on peptide or phosphosite intensities.

The combined data shows that the trap-elute micro-flow setup on the ZenoTOF 7600 performs well for proteomics, identifying similar numbers of proteins in a 30-minute Zeno SWATH run using a 400 ng load compared to a 90-minute SWATH run with a 2 µg load on a TripleTOF 6600. Future work should focus on optimising the workflow for Zeno SWATH phosphoproteomics to increase identifications.

id #89608

An investigation of the physiochemical changes in field pea flour after heat treatment.

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Field peas are a nutritious food source that have shown potential as a functional food ingredient and heat treatment can be used to improve the sensory and functional characteristics of the flour. Changes to the pea proteome before and after heat treatment can be monitored using MS-based techniques, however plant proteins exhibit poor solubility and require an effective extraction protocol in the LC-HRMS (Orbitrap) workflow for satisfactory sequence coverage and protein ID confidence. Evaluation of three unique extraction protocols 50% (v/v) isopropanol (IPA) 50 mM Tris-OH, and a 2-step IPA → 8 M urea protocol (Combo) showed IPA extracted 8.6% protein, Tris-OH 40.6% and Combo 87.1% from raw pea flour (17.2% protein). SDS-PAGE showed IPA enriched low MW proteins (<20 kDa) while Tris-OH and Combo showed clear bands from 20-100 kDa, with Combo depleted of proteins <20 kDa. The number of proteins/peptides identified using LC-HRMS by each extraction protocol were 356/1781 (IPA), 431/2824 (Tris-OH) and 421/2707 (Combo). Heatmap and PCA analysis showed IPA extraction favoured proteins involved in nutrient storage, plant defence and metal ion binding, Tris-OH extracted more cytosolic proteins involved in amino acid synthesis and energy production while Combo tended to extract cell membrane proteins with a higher isoelectric point but did not enrich any specific class. Tris-OH was selected for subsequent proteomic analysis of heat-treated flour samples. To evaluate the effect of heating on the pea flour, samples were heated in a microwave for 5 min or drum roaster for 7 min. SDS-PAGE showed no major changes in protein profile following microwave roasting however, drum roasting showed evidence of protein aggregation and degradation of protein. LC-HRMS analysis showed a significantly lower protein ID count after either heat treatment and a significantly higher missed cleavage rate after drum roasting. Analysis of protein bound Maillard reaction products (MRPs) revealed that microwave roasting caused a ~4-fold increase in modified peptides and drum roasting an ~11-fold increase when compared to the untreated flour. Modifications were predominantly methylglyoxal derived hydroimidazolones (MG-H) within high abundance nutrient storage proteins vicilin and legumin. Proteomic approaches provide a relatively fast and effective tool for determining the need for further quantitative mass spectrometry of MRPs and providing the plant-based food industry with insight into the effect of different heat processing techniques.

id #89144

Shotgun proteomics and JESS Simple Western analysis to characterize kisspeptin-induced Mesenchymal-Like Transition mechanism in KISS1R-expressing Glioblastoma

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The mesenchymal-like transition in Glioblastoma (GBM) can increase the tumor cell motility, promoting its high degree of infiltration into the surrounding brain region. This often compromises the maximal tumor resection, leaving the residual foci that lead to recurrence. Kisspeptin, a neuropeptide that acts via G protein-coupled receptors (GPCRs), KISS1R (GPR54) has been reported to regulate the mesenchymal-like transition process in cancers. Preliminary data demonstrated kisspeptin (10 – 100nM) induced cell scratch closing with observed morphological changes, hypothesizing its role in influencing cell motility via mesenchymal-like transition in KISS1R-expressing GBM cells. Therefore, this study aims to characterize the underlying cell motility via a mesenchymal-like transition mechanism utilizing the shotgun proteomics and JESS Simple Western analysis in KISS1R-expressing GBM.

The kisspeptin effect on cell proliferation was performed using CCK-8 kit. Following this, the mesenchymal-like transition was conducted by immunocytochemistry (ICC) analysis using rhodamine phalloidin (3 - 48 hours). The mechanistic data was evaluated by proteomic analysis with liquid chromatography-mass spectrometry (LC-MS/MS), where the identified molecular markers and mechanisms were further validated using JESS Simple Western.

CCK-8 analysis demonstrated that kisspeptin did not affect cell proliferation and viability in GBM, indicating that the treatment effects observed in kisspeptin-treated cells were not due to the cell proliferation and stress-mediated effects. The confocal ICC analysis showed that kisspeptin treatment (10 and 100 nM) induced mesenchymal-like transition in KISS1R-expressing GBM, as early as 3 hours and sustained up to 48 hours. The initial proteomics (LC-MS/MS) analysis confirmed that the KISS1R-expressing GBM cells are not kisspeptin-producing cells. Furthermore, the shotgun proteomics profiling revealed the regulated expression of mesenchymal-related markers (vimentin), including those of cell adhesion molecules, cytoskeletal proteins and matrix proteases, including the upregulation of transgelin-2 and cofilin, following kisspeptin treatment in the KISS1R-expressing GBM. JESS Simple Western confirmed kisspeptin treatment upregulated the KISS1R expression, indicating KiSS/KISS1R system mediated-mesenchymal transition. Moreover, the data demonstrated the upregulation of mesenchymal marker (N-cadherin) and transcription factors (ZEB-1, Slug, Snail and β-Catenin).

The current data suggested that kisspeptin induced a sustained mesenchymal-like transition process (up to 48 hours), whereby the shotgun proteomics and JESS further characterize the mechanisms via classical mesenchymal markers and transcription factors regulation. This warrants the utilization of LC-MS/MS and JESS for future comprehensive mesenchymal-like transition mechanisms by kisspeptin that may fill the knowledge gap of GBM infiltrative nature.

id #89178

The phospho-regulation of histone demethylases in *Saccharomyces cerevisiae*

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Histone methylation is a dynamic regulator of transcription that has been correlated with both gene activation and gene silencing (1,2). Dysregulation of the histone methylation system has been linked to the aetiology and progression of several diseases, including cancer and neurodegenerative disorders (1,2). In the model organism, *Saccharomyces cerevisiae,* histone methylation is mediated by a total of eight evolutionarily conserved methyltransferases and demethylases (3-6). There is emerging evidence that histone methylation itself is regulated by the kinase signalling network via phosphorylation (7,8). Despite this, the function of many phospho-sites on histone methylation enzymes – particularly demethylases – in *S. cerevisiae* is still unknown. Consequently, we seek to investigate the phospho-regulatory system of histone demethylases in *S. cerevisiae*

and its role in modulating cellular response phenotypes. To start, we conducted a comprehensive analysis of the literature to identify candidate conditions in which demethylase knock outs have shown abnormal growth phenotypes. Using spot plate growth assays, we then showed that almost all known *S. cerevisiae* demethylase kockouts (Rph1p, Jhd1p and Jhd2p) had clear phenotypes in at least one condition tested. In particular, the H3K36 demethylase, Rph1p, was found to be essential to the galactose genetic switch. However, subsequent analysis of H3K36 methylation using liquid chromatography tandem mass spectrometry (LS-MS/MS) showed no significant changes in global methylation when grown in media supplemented with galactose instead of glucose. This suggested that changes in H3K36 methylation were probably localised to specific regions of the chromatin (ie. the regulatory GAL genes). To determine whether this is the case, we intend to measure changes in methylation of K36 at histone H3 bound at specific genes/coding sequences under these conditions. Our future work will then look to link observed changes in histone methylation to changes in Rph1p phosphorylation at specific sites using a combination of phospho-site mutants and LC-MS/MS analysis of purified Rph1p expressed under the same conditions. Ultimately, our aim is to identify the kinases that act on candidate phospho-sites to induce changes in gene expression and cellular phenotypes. This research will allow us to identify which histone methylation regulatory pathways may be conserved across eukaryotes, as well as identify those that are unique to yeast.

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id #89627

Chemoresistant Cancer Cell Lines Are Characterized by Migratory, Amino Acid Metabolism, Protein Catabolism and IFN1 Signalling Perturbations

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Chemoresistance remains the major barrier to effective ovarian cancer treatment. The molecular features and associated biological functions of this phenotype remain poorly understood. We developed carboplatin-resistant cell line models using OVCAR5 and CaOV3 cell lines with the aim of identifying chemoresistance-specific molecular features. Chemotaxis and CAM invasion assays revealed enhanced migratory and invasive potential in OVCAR5-resistant, compared to parental cell lines. Mass spectrometry analysis was used to analyse the metabolome and proteome of these cell lines and was able to separate these populations based on their molecular features. It revealed signalling and metabolic perturbations in the chemoresistant cell lines. A comparison with the proteome of patient-derived primary ovarian cancer cells grown in culture showed a shared dysregulation of cytokine and type 1 interferon signalling, potentially revealing a common molecular feature of chemoresistance. A comprehensive analysis of a larger patient cohort, including advanced in vitro and in vivo models, promises to assist with better understanding the molecular mechanisms of chemoresistance and the associated enhancement of migration and invasion.

id #89164

Use of DIA-MS to determine the effects of storage temperature and time on FFPE tissue sections

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Formalin-fixed paraffin-embedded (FFPE) tissues are invaluable resources for cancer proteomic studies and biomarker discovery. Very large numbers of them are stored in pathology laboratories and biobanks worldwide and often have associated clinical data available. FFPE tissue blocks are stable for decades when stored at room temperature (RT) and can be sectioned to produce samples for liquid chromatography-mass spectrometry (LC-MS) analysis. It is not known if the proteome of FFPE tissue after sectioning may be affected by storage temperature or time. To address this, we stored FFPE tissue sections at RT and -80°C for up to 336 days and analysed them at different timepoints to determine the proteome stability. A total of 297 FFPE 10 μm sections (triplicates of rat brain, kidney and liver) were cut from tissue blocks and stored at either RT or -80°C. Control samples were freshly cut sections from FFPE blocks. Samples were prepared for LC-MS analysis by digestion at 11 timepoints after storage (up to 336 days) and analysed by microflow HPLC on Triple TOF 6600 mass spectrometers (SCIEX) using data-dependent acquisition (DDA) mode. Kidney and liver digests were further analysed in data-independent acquisition (DIA) mode for quantitative analysis. Nine post-translational modifications (PTMs) that are specific for FFPE samples were identified from the DDA data analysis. These PTMs were monitored for quantitative changes in samples that were stored at different temperatures and for different time periods using DIA data. Overall, the storage temperature and time did not have significant effects on the proteomic analysis of FFPE sections. After FFPE blocks are cut, the tissue sections can be safely stored at either RT or -80°C for at least 1 year without significant impacts on the identified proteome. Such samples are suitable alternatives for fresh frozen tissues in proteomic studies.

id #89436

Analysis of cellenONE sorted and prepared single cells in a label-free nano-flow LC-MSMS proteomics approach using a nanoElute 2 and an Evosep One system and a timsTOF SCP

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Introduction

Single cell proteomics is a rapidly developing field with the potential to make important contributions to the understanding of cellular heterogeneity. Single cell protein extraction, minimal exposure of samples to surfaces and optimal storage and transfer conditions are crucial for loss-less single cell proteome analyses. Recent enhancements in trapped ion mobility spectrometry (TIMS) coupled to fast and sensitive mass spectrometry established in the timsTOF SCP paired with automated single cell sorting and sample preparation realized with the cellenONE® platform allows for sensitive proteome analyses at the single cell level.

Methods

Single HeLa cells were sorted into the label-free proteoCHIP®, directly lysed, and proteins digested at 50°C with high humidity on deck using the cellenONE platform. The label-free proteoCHIP with tryptic peptides was placed into the nanoElute 2 autosampler, peptides injected onto a 25 cm Aurora C18 column (IonOpticks) and eluted into a timsTOF SCP. cellenONE Sorted and prepared single HeLa cells were loaded onto Evosep Pure tips and analyzed with an Aurora Elite column at 50 °C using the Whisper 40 SPD method and a timsTOF SCP. dia-PASEF® mode was used and analyzed with TIMS-DIA-NN on PaSER® using a library generated from a deeply fractionated human cell line.

Results

Sample pick-up directly from the label-free proteoCHIP was assessed with HeLa lysate digests (Pierce) showing excellent reproducibility at various concentrations. Injections of 250 pg of HeLa peptides on column (1 μL in well) resulted in 15,000 peptides from 2,600 proteins which was matched by 250 pg HeLa peptides injected from a vial (250 pg/μL). We then analyzed single HeLa cells which were directly sorted and prepared in the label-free proteoCHIP and identified in average more than 2000 proteins per single cell with good reproducibility.

Further, we analyzed a HeLa peptide dilution series ranging from 62.5 pg to 32 ng (n = 6) loaded onto Evptips Pure. From the 250 pg load, we identified 12,000 peptides from 2,500 proteins and from the 32 ng load 70,000 peptides from 7,000 proteins with excellent reproducibility. Sorted single HeLa cells prepared in the label-free proteoCHIP were loaded onto Evotips Pure and analyzed in Whisper 40 SPD leading to more than 10,000 peptides and 2,000 proteins identified from a single cell.

Conclusion

Label-free analysis workflow that reproducibly identifys >2000 proteins from single HeLa cells, using the cellenONE platform with the lable-free proteoCHIP, nanoElute 2, Evosep One and the timsTOF SCP.

id #89287

Chemoproteomic Target Deconvolution Approaches in *Giardia duodenalis* Using Kinase Inhibitors

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Giardia duodenalis is a gastrointestinal parasite causing ~200 million symptomatic infections annually, disproportionately affecting children in lower socioeconomic tiers where it causes malnutrition, stunting in physical and cognitive growth. Chemotherapeutic treatments for such infections are limited to nitroheterocyclic antibiotics such as metronidazole. However,

high doses of these drugs are toxic and treatment failure related to drug-resistance occurs in up to 20% of cases. This highlights a need for a novel and safer chemotherapeutic option for treating *Giardia* infections.

The *Giardia* kinome is an attractive druggable space, as the NEK (Never in Mitosis A related kinases) family of kinases have been expanded in this genus' otherwise reduced kinome (198/278 of annotated kinases). We performed a medium-throughput *in vitro* screen of a published kinase inhibitor library and identified a putative compound class that is effective against *Giardia* at sub-micromolar concentrations. As a next step, we have undertaken drug-target identification using the Proteome Integral Solubility Alteration (PISA) assay and affinity-chromatography "pulldown" with MS-based identification. In the former, we shortlisted 63 proteins demonstrating dose-dependent thermal-(de)stabilisation resulting from ligand binding. Four of these 63 proteins were classified as pseudokinases (3 NEKs), presumed catalytically inactive kinases that are the likely primary targets of our compound. These will be cross-referenced with hits generated through our pulldown dataset. Further, *in silico* binding predictions of our lead compound with the four kinases identified in PISA produced comparable ligand-protein intermolecular interactions to those described in the literature. Together, these orthogonal approaches have allowed us to identify credible candidate targets for the compound in *Giardia*, which we will now explore through target-based assays and additional compound chemistries.

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id #89365

Defining the Structures and Interactions of the Human Platelet Secretome using Chemical Crosslinking

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Platelet activation induces the secretion of proteins that promote platelet aggregation and inflammation. However, the understanding of the regulation and function of these proteins, many of which are only expressed in platelets, has been hampered by a lack of tertiary structure information and limited knowledge of protein-protein interactions. Here, we detail the first chemical crosslinking analysis of the platelet secretome (releasate) using DSSO and nanoLC-FAIMS-MS2 analysis with stepped-HCD fragmentation. Platelets from 3 different patients were each stimulated by thrombin treatment to release their granule contents. These secreted proteins were crosslinked with an optimised DSSO concentration (0.1 mM) and after trypsin digestion were fractionated by offline high pH reversed-phase chromatography to increase crosslinked peptide coverage. MaxQuant was used for crosslinked peptide identification as it showed significant sensitivity improvements compared to XlinkX. Across the 3 patients 1,844 crosslinked peptides were identified from 143 different secreted platelet proteins. For analysis of platelet proteins without confirmed tertiary structures, we generated our own structural predictions with Colabfold (Alphafold2) incorporating knowledge of each protein's multimerization state. The distance constraints from our crosslinking data were then mapped onto these predicted structures. In general, the crosslinking distance constraints confirmed the predicted structures, which opens the door for these structures to be used for further functional interrogation of these proteins. Regarding protein-protein interactions derived from the interprotein crosslinks we identified, many previously known positive controls were observed such as fibrinogen alpha-beta-gamma trimers. However, many new interactions were also observed and will be the subject of subsequent confirmatory experiments by IP-MS and other methods. One of the unexpected findings was excellent crosslinking data for HDL particles, which are known to associate with platelets. We observed many crosslinks between APOA1 and APOA2, which allowed us for the first time to propose a structure of this key protein complex. The comprehensive human platelet crosslinking dataset provided here will allow identification of novel regulatory mechanisms for drug targeting to address platelet dysfunction and thrombosis.

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id #89140

A complete pipeline for human gut microbiome meta-proteomics study

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The gut microbiome is linked with obesity through altering nutrient absorption, pathogen resistance and inflammation through modulating the immune system, as well as neurodegenerative diseases through endocrinological signalling pathways. As such, meta-proteomics aims to study the proteome of these gut microbes in their host environment, which has been shown to be crucial for our understanding of human health.

However, analysing meta-proteomic data requires the capability to handle large-scale datasets containing qualitative and quantitative information of proteins identified from highly redundant databases. \Although many approaches are available for conventional proteomics analysis, these still have difficulties processing large chimeric databases for meta-proteomics studies. To address these issues, we established a workflow combining quantitative proteomics methods such as tandem mass tag (TMT) quantification or label-free quantification (LFQ) with sample-specific databases refined from both the host as well as the microbes. This significantly reduces analysis time without sacrificing the quality of the outcome of a meta-proteomics study. Herein we present two representative case studies displaying our capabilities in human meta-proteomics with an example of label-free and label-based workflows.

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id #89152

Development of a Novel Trabecular Bone Tissue Microarray for 20µm Resolution *N*-Glycan MALDI Mass Spectrometry Imaging

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Publish consent withheld

id #89184

Simulation of DIA mass spectrometry proteomics data with Synthedia

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Comprehensive identification and accurate quantitation of peptides is the key goal of mass spectrometry-based proteomics. To achieve as complete data as possible, researchers need to configure a large number of settings and parameters when conducting both experimental analysis of samples as well as bioinformatic processing of the raw data. Importantly though, these instrumental and bioinformatic processing parameters do not operate in isolation and a favourable change in one parameter frequently has a detrimental impact on the experiment elsewhere. Optimising an experimental and bioinformatic analysis pipeline involves finding a balance between parameters that give acceptable results and a substantial literature has been produced in support of this endeavour.

When optimizing experimental methods, researchers typically analyse real experimental samples under a variety of conditions and then process the raw data with a range of search software and parameters. Key metrics generated by these analyses are the qualitative list of proteins and peptides identified and relative abundance values for each species. However, when different instrumental analysis methods and/or different computation analysis software give conflicting results, the 'best' option can be difficult to select. In part, the challenge in selecting an optimal method is that the 'true' complement of peptides and proteins present in a complex sample is never completely known and researchers don't have a ground-truth against which the results of optimisation processes can be compared.

Here, we present Synthedia – a computational platform that can generate DIA-LC-MS data files *in silico* in mzML format with a complement of peptide ions and fragments that is exactly known. A wide range of different operating parameters can be simulated such as varying gradient lengths, chromatographic peak widths, scan speeds, mass spectral resolutions and isolation windowing schemes and experiments can be simulated that contain multiple 'treatment groups' and 'replicates'. To demonstrate the use of this software, we conduct extensive simulations of data with different acquisition speeds to demonstrate how quantitative accuracy declines with decreasing points per chromatographic peak. Lastly, we demonstrate how rates of peptide identification, false-positives and false-negatives vary between different DIA data anlaysis software as a function of both chromatographic peak width and chromatographic gradient length.

id #87724

Linear models and empirical Bayes methods for proteome-wide label-free quantification and differential expression in mass spectrometry-based proteomics experiments

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Mass spectrometry-based proteomics is a powerful tool in biomedical research, but its usefulness is limited by the frequent occurrence of missing values in peptides that cannot be reliably quantified. Many analysis strategies have been proposed for missing values where the discussion often focuses on distinguishing whether values are missing completely at random (MCAR), missing at random (MAR) or missing not at random (MNAR). We argue that missing values should always be viewed as MNAR in label-free proteomics because physical missing value mechanisms cannot be identified for individual points, and because the probability of detection is related to the underlying intensity. We propose a statistical method for estimating the detection probability curve as a function of the underlying intensity, whether observed or not. The model demonstrates that missing values are informative and quantifies the bias of missing intensities as compared to those that are observed. The distribution of missing intensities is estimated from the observed values on the peptide level, following which a new protein-level quantification method by linear models is introduced. The empirical Bayes method in limma is also revised to account for uncertainty caused by imputed values. Performances of the proposed pipeline are evaluated on real proteomics data sets with the mixture design, where two distinct samples are mixed in known proportions. We show that the proposed method eliminates missing values in protein-level quantification and improves the statistical power for differential expression in proteome-wide experiments.

id #89228

Supercharge Your Immunopeptidomics

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Human leukocyte antigen (HLA) molecules play a crucial role in the development of adaptive immune responses and therefore have been of major interest in the development of immunotherapies such as cancer vaccines and chimeric antigen receptor (CAR) T cells. Hence, a comprehensive understanding and profiling of the immunopeptidome is required to foster growth for these personalised solutions. There are however several analytical challenges associated with immunopeptidome profiling, as highlighted by the Human Immunopeptidome Project Consortium. These include (i) uncertainties around peptide isolation, (ii) the inability to analyse small amounts of biological material, (iii) low throughput, (iii) lack of sensitivity and reproducibility of mass spectrometry technologies, (iv) suboptimal identification rates, (v) lack of experimental and computational standards, and (vi) the current lack of accessibility to large-scale community generated datasets.

We herein describe a novel immunopeptidomics workflow involving the Kingfisher platform (Thermo Scientific) to isolate immunopeptidomes with anti-HLA antibodies coupled to a proprietary hyper-porous protein A microparticle (MagReSyn®), and an improved LC-MS/MS method using dimethyl sulfoxide (DMSO) as supercharging agent.

Using this workflow, we were able to identify up to ~5000 and ~7000 unique HLA class I and class II peptides, respectively, in as low as 5e6 B lymphoblastoid cell line. Moreover, addition of 5% DMSO to the LC-MS/MS buffer systems was observed to increase the number of HLA-bound peptides by ~50%. Our data strongly suggests that this increase is mostly due to the presence of higher quality ms2 spectra showing a significant increase in b and y ions intensities which result in improved signal-to-noise ratios (~1.5 and ~2 fold for HLA class I and class II bound peptides).

Altogether, this improved workflow will be very important for the future of the immunopeptidome profiling, especially for smaller sample types, and ultimately can be a useful tool for the development of immunotherapies and other precision medicine approaches.

id #89449

Yeast secretome response to the ER stress

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Endoplasmic Reticulum (ER) stress happens when the capacity of the ER to fold proteins is overwhelmed. There are many causes of ER stresses, including mutation of genes involved in glycosylation such as $\Delta ost3$ and $\Delta pmt1$, the addition of antibiotics or redox-active chemicals such as Tunicamycin or DTT, as well as thermal perturbation. These stresses cause defects in N-glycosylation, O-glycosylation, or disulfide-bond formation, and consequently activate ER stress signals. Our study profiled the proteomes secreted from yeast under different ER stress conditions and successfully identified secretome differences in both the type and abundance of secreted proteins between different ER stress conditions based on SDS-PAGE analysis and SWATH-MS analysis. Our results are relevant to understanding the physiological responses of eukaryotes to diverse stress conditions and in industrially relevant settings including yeast fermentation for beer and wine production.

id #89292

Disruption of fungal cell wall carbohydrates modulates EV protein load in a pathway specific manner.

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Fungi occupy an incredibly diverse range of biological niches, including symbiotic, commensal, and pathogenic relationships with other organisms. The cell wall of the fungus is the first point of contact and the major mediator of these interactions. Research strategies to control fungal disease and boost beneficial relationships must account for cell wall biosynthesis and remodelling. We hypothesised that extracellular vesicles (EVs) and non-classical secretion pathways are essential for cell wall remodelling during the response to cell wall perturbation in fungi. In this study, extracellular vesicles of the model yeast *Saccharomyces cerevisiae* were analysed using LC-MS bottom-up proteomics to assess the impact of gene knockouts in key cell wall biosynthetic pathways. Independent gene deletions affecting the major cell wall carbohydrates 1,3-beta-glucan and chitin mimicked the effect of antifungal strategies and allowed assessment of cell wall remodelling and the response to these interventions. Disruption of chitin biosynthesis resulted in increased EV load for proteins related to cellular localisation and transport and decreased abundances for primary metabolic pathways. Disruption of 1,3-beta-glucan biosynthesis saw an increased EV load of cytosol-annotated proteins and a loss of EV proteins required for bud formation and cell division. These findings suggest disruption of cell wall biosynthesis in the fungi exerts unique influences on EV proteins dependent on the structural carbohydrate.

id #89273

Selective washing of dried blood spots provides enhanced coverage and quantification of the blood proteome

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The microsampling of blood in the form of dried blood spots has several advantages: the convenience of sample collection (small volume and no special equipment or personnel required); the ease of storage and transport; the accessibility of at-home and remote community sampling; and the application of these to longitudinal studies. However, analysis of blood for proteomics using mass spectrometry has always been limited due to the very wide dynamic range. This is most often overcome by utilising multiple offline and online fractionation which can achieve improved coverage. These approaches can produce thousands of protein identifications however, they are not well suited to scale-up for large number of samples. Using small volume (30 µL) volumetric absorptive microsampling (VAMS) devices we have developed a series of methods to fractionate samples via the use of various wash solutions and in-tip digests. These methods have been found to successfully decrease the dynamic range of dried whole blood and enhance detection of the blood proteome.

We present here various wash buffer combinations (salts, organic solvents, acids, detergents) and demonstrate (using DIA-MS) that we can quantify greater than 3,500 proteins from whole blood and 1,000 proteins from plasma. Each wash produces a distinct protein profile and hence can be used to target specific protein and/or peptide populations. We also demonstrate that our methods enable extraction using solutions typically incompatible with mass spectrometry analysis (i.e. CHAPS) to produce unique and comprehensive datasets without compromising data quality. The flexibility of these methods enables multiple extractions from a single tip for multiplexing of different analyses (i.e. extraction for immunochemistry assay followed by extraction and digestion for mass spectrometry) and they are applicable to a wide range of sample types including whole blood, plasma, peripheral blood mononuclear cells (PBMCs), red blood cell pellets, saliva, and cell lines.

These methods and results outline a new approach to sample preparation that can help increase protein identifications from small volumes, complex protein solutions, and samples containing SDS or CHAPS. Overall, the ability to get excellent coverage and reproducible protein extraction from VAMS devices using fractionation will further enable their use in protein biomarker studies.

id #89392

Comparative Glycoproteomics Analysis of Different Treatments to Induce Yeast Autolysis in Sparkling Wines

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Sparkling wine is an alcoholic beverage that is enjoyed globally. It commands an intoxicatingly high-quality niche within the highly competitive wine market. While the *Méthode Traditionelle* is the most popular method in producing high quality sparkling wine, new techniques have been developed with the aim to artificially induce yeast autolysis, thereby reducing the required time to ferment and produce high quality sparkling wine. Samples from Chardonnay and Pinot Noir sparkling wines, each with a different treatment or condition, have been prepared and analysed by LC-ESI-MS/MS, with the resulting data being analysed via GlypNirO's SWATH pipeline, as well as an ion library assembled using DDA files of glycopeptide identifications searched in Byonic, to aid with the SWATH/DIA analyses. From there, a comparative analysis, of the treatment options currently present, have been conducted on Chardonnay and Pinot Noir sparkling wines in order to determine which treatment options are more effective in accelerating yeast autolysis without negatively affecting the sparkling wine's organoleptic qualities.

id #89375

The unfolded protein response and dynamic host subcellular proteome in response to H1N1 and H3N2 influenza virus infection

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Influenza viruses, particularly influenza A virus (IAV), are responsible for seasonal and pandemic outbreaks of Influenza, a respiratory disease which heavily burdens the elderly population. Two subtypes of IAVs are commonly transmitted amongst humans, the H1N1 and H3N2 subtypes. Seasonal outbreaks that are dominated by the H3N2 virus tend to be more severe. Due to both the emergence of antiviral resistance, and the lack of a universal vaccine for influenza viruses, developing our understanding of viral-host protein interactions remains essential. Although global proteomic changes in influenza infection have previously been investigated, little attention has been given to characterising these changes at a subcellular level or with consideration to protein post translational modifications such as glycosylation. We used a subcellular fractionation protocol to isolate cellular compartments relevant to influenza virology: the nucleus, cytoplasm, organelle, and secreted proteins. We developed an LC-MS/MS approach combining tandem mass tag (TMT) labelling and hydrophilic liquid interaction chromatography (HILIC) enrichment of glycopeptides. Using this approach, we are able to quantify and compare protein and glycoprotein abundances in each fraction throughout a time-course infection of human A549 cells with two influenza A strains: the H1N1 virus strain A/Puerto Rico/8/1934, and the H3N2 strain A/X-31. Here, we demonstrate the dynamic subcellular host response to IAV infection and describe strain-specific responses observed in relation to the unfolded protein response (UPR).

id #89402

Integrating Protein Correlation Profiling with Cross-Linking Mass Spectrometry to build a comprehensive map of protein-protein interactions in the malaria parasite

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The *Plasmodium* parasites that cause malaria represent a branch of eukaryotic life that has been committed to an intracellular parasitic lifecycle for as much as a billion years. In this time they have evolved a highly specialised and divergent biology to accommodate a complex lifecycle spanning multiple host cell types across insect and vertebrate hosts. Despite their place as one of the most important human pathogens, much of this biology remains poorly understood. For example, a third of protein-coding *Plasmodium* genes lack any form of functional annotation, a further third are only putatively annotated on the basis of often limited homology to characterised proteins in other species. Because of this, existing interventions to treat malaria and to control its spread share a limited range of molecular targets, and are increasingly threatened by the emergence of resistance. To address this lack of knowledge, we are applying Protein Correlation Profiling (PCP) and Cross-Linking Mass Spectrometry (XLMS) to *P. falciparum*, with the goal of establishing a comprehensive map of protein-protein interactions in this species. PCP and XLMS have recently emerged as highly complementary approaches to the study of protein interactions at proteome scale, but they have not been widely applied together, or outside of model organisms. We have developed new machine-learning protocols to enable the integration of our PCP and XLMS datasets with existing data, resulting in a complexome that currently covers almost half of the observable *P. falciparum* proteome. Our complexome confirms the presence of a range of conserved eukaryotic protein complexes, including several that have not previously been experimentally characterised in *Plasmodium*. We also identify *Plasmodium*-specific complexes, as well as conserved eukaryotic processes that appear to be divergent in *Plasmodium*. For example, we illuminate the unusual composition of the *P. falciparum* CMG helicase that initiates DNA replication in eukaryotes and archaea. Work is ongoing to further validate and expand the *P. falciparum* complexome, and to exploit its insights to better understand parasite biology and to identify new therapeutic targets.

id #89260

Proteomic profiling of small extracellular vesicles isolated from an *in vitro* cell culture bioreactor simulating *Mycoplasma bovis* infection

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Mycoplasma bovis (*M. bovis*) is a pathogenic bacterium causing untreatable mastitis, abortions, swollen joints, and severe arthritis in cattle of all ages. Though eradication efforts are ongoing, they are mostly limited by ineffective antibiotics and suboptimal diagnostic tests. In recent years, small extracellular vesicles (sEVs), and exosomes in particular, have gained significant interest. Exosomes are small extracellular vesicles of 30-150 nm known to have important functions in cellular communication, signal transduction, and immunomodulation. As these nanoparticles contain proteins, lipids and metabolites, representative of their cell of origin, their content may have potential to carry biomarkers for diagnosis of early or hidden infections.

In this work, we want to unlock the diagnostic potential of sEVs isolated from control cells vs *M. Bovis* infected cells. We hypothesize that comparing their exosomal proteome may contain information representative of infection. To this end, a control culture of a bovine endometrial epithelial cell line (bEEL cells) (n = 5) and a co–culture of bEEL cells and *M. bovis* (n=5) were established within bioreactor flasks. From each bioreactor harvest, sEVs were isolated using Size Exclusion Chromatography qEV10 columns (IZON, New Zealand). The isolated exosomal fraction was further heat treated for 5 min to inactive *M. Bovis* before proteins were extracted and digested.

A nanoElute UHPLC was coupled online to a Bruker timsTOF Pro 2. Peptides were separated on a reversed-phase column (25 cm x 75 µm i.d.), packed with 1.6 µm C18 silica beads (IonOpticks, Australia) using a 60 min linear gradient from 2 to 35% B (0.1% formic acid/acetonitrile/water). Data were acquired using diaPASEF, with data independent isolation of multiple precursor windows within a single TIMS separation (100 ms). For each sample, data were acquired in duplicate. Analysis of the 4D data space was performed using PEAKS Online, combining a spectral library and database search.

In total, over 4100 proteins representing 2816 protein groups were identified across the samples. As expected, hundreds of mycoplasma proteins have been identified in the sEVs of the co-cultures, but not in the control samples. Additional comparative proteomics profiling of the altered bovine proteome of sEVs in response to infection by *M. bovis* indicated separate grouping. Specific exosomal proteins were detected using ExoCarta. In conclusion, our data provides preliminary evidence that exosomal protein profiles are altered in the presence of *M. bovis* infection and can thus play a crucial role in early diagnosis of the disease.

id #89170

Stability of cytokines in dried blood spots collected using volumetric absorptive microsampling (VAMS) and stored at various temperatures.

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Dried blood spots (DBS) collected on filter paper such as Guthrie cards are stored for years at room temperature. The supposition is that once dried, the samples remain stable and quantifiable indefinitely since the metabolites these were initially designed to measure, are known for their extended stability. The concentration of other blood proteins such as cytokines, however, are known to vary with storage even in liquid samples stored at -80 °C for extended periods of time. Thus, we sought to determine if cytokines are stable for up to 5 months when stored as a dried blood spot.

To test this, blood was collected from 4 healthy participants, spiked with recombinant cytokines, and collected into 30 µL volumetric adsorptive microsampling (VAMS) devices. These prepared DBS were stored at room temperature, 4 °C, or -20 °C for up to 5 months and matching DBS liquid extracts were stored at -80 °C for the same period of time. At each timepoint, the DBS were extracted from the VAMS and the extracts were analysed by Luminex® for quantification of up to 31 cytokines.

Cytokine analysis revealed that room temperature, the current standard for DBS storage, performed the poorest out of all storage temperatures with 9/21 significantly different to baseline by 5 months compared with 4/21 for the 4 °C comparator. Storage at 4 °C or colder performed well for the majority of analytes tested, however out of those, the optimal storage temperature differed for each analyte. For example, IL-21 was highly variable at 4 °C and more reproducible at -80 °C, whilst the opposite was true for IL-13. There were a small number of analytes that performed poorly regardless of storage conditions and for fractalkine, this was found to be caused by inefficient recovery during extraction.

Our results highlight the need to understand the stability of analytes of interest before committing to longitudinal collection and storage of DBS. These data give confidence that with storage at 4 °C or colder, 27/31 of the cytokines assessed in this study are quantifiably stable over for at least 5 months.

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id #89282

ATLANTIS – ATLas of peptide ANtigens in Transplantable tISsues

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Transplantation is a life saving procedure for patients with end stage diseases, traumatic injuries, blood diseases or metabolic disorders. For solid organs or tissues the most common type of transplantation performed is allogenic grafts that contain genetic mismatches between the human leukocyte antigen (HLA; human major histocompatibility complex [MHC]) molecules carried by the donor and recipient. HLA mismatches can trigger destructive immune responses leading to graft rejection and loss. Recipient T cells that mediate these immune responses recognise foreign donor HLA molecules in complex with self-peptides (1). A major limitation following transplantation has been an inability to identify the peptide/HLA targets involved in graft rejection and the alloreactive T cells that directly recognise them. We recently reported in mice that the donor immunopeptidome (array of peptides presented by MHC molecules) provides strong immunogenic targets for recipient alloreactive T cells that mediate anti-graft responses. Importantly, we have shown that as few as 5 different peptide antigens commonly expressed by three different tissues (i.e. hepatocytes, spleen, skin) trigger ~40% of alloreactive T cells, demonstrating that there are immunodominant allogeneic peptide/MHC complexes that account for a large proportion of the anti-graft response (2). In this study, we explored the human immunopeptidome of transplantable organs (i.e. heart, lung, kidney, liver) from 5 HLA-A*02:01-positive donors to profile both commonly expressed and tissue-specific peptides presented by a globally highly prevalent HLA allotype. Briefly, 100-300 mg of tissue was used for the immunoaffinity purification of peptide/HLA class I and class II complexes and the peptides isolated for analysis by LC-MS/MS on a Bruker timsTOF Pro2. Data were searched with PEAKS Xpro studio software against the Homo sapiens UniProt database. Matched tissue-specific proteomics data is also available from each sample. We anticipate that the ATLANTIS database will allow for the identification of donor-derived peptide antigens that drive anti-graft immune responses, which will be critical for future development of diagnostic reagents for post-transplant immune monitoring.

1. Felix et al. Nat Immunol 8, 388-397 (2007).
2. Son et al. J Clin Invest Nov 1;131(21):e146771 (2021).

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id #89413

Statistical methods and machine learning in the diagnosis of endometrial cancer

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Classic histopathological examination of tissues remains the mainstay for cancer diagnosis and staging. In some cases, histopathologic analysis yields ambiguous results, leading to inconclusive disease classification. Here, we set out to explore the diagnostic potential of mass spectrometry-based imaging for tumour classification based on proteomic fingerprints. Supervised machine learning (ML) approach was applied to large MALDI MSI datasets from endometrial cancer (EC) TMAs consisting of 302 unique patients. The pathologist labels for these patients included 43 patients of primary tumour with lymph node metastasis (LNM), 214 patients with no LNM, and 45 patients that were unclassified/not tested. Combining mass spectrometry

with ML, we were able to predict the presence of LNM in primary tumour of EC with an overall accuracy of 80% (90% sensitivity and 69% specificity). In addition, this approach was able to distinguish colorectal tumour from normal tissue with an overall accuracy of 98% (98.2% sensitivity and 98.6% specificity). Using supervised ML of EC MALDI MSI data, in conjunction with pathologist annotation and patient meta data, this study set to establish approaches for binary sample classification problems. This is achieved based on the MALDI MSI data alone, without any need to identify the m/z values. Overall, these results highlight the potential of this technology to determine the optimal treatment for cancer patients to reduce morbidity and improve patients' outcomes.

id #89237

Scaling proteomics to population size cohorts: Early insights and opportunities from ASPREE proteomics

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Proteomics analysis of biofluids has a demonstrated potential to be a rich source of disease biomarkers. Despite the numerous case-control studies in biofluid proteomics, clinical translation from bench to bedside remains poor. While large-scale population proteomics offer greater potential for clinical translation, technical challenges and inherent variability of LC-MS systems have hindered further progress in the field. Here, we present our journey to develop a robust pipeline for scalable proteomics using highly controlled sample and data processing workflows. Using a novel data-driven experimental design, we are in the process of producing the largest proteomics dataset ever-generated, profiling two timepoints of >12,000 urine samples from the ASPREE cohort with an average over 10,000 peptides per sample. We present an exploratory analysis of a data subset of more than 8,000 MS runs across 20 batches, focusing on consistency, coverage, within and across batch variability. We demonstrate the great potential of the resource in addressing predictive biomarker discovery for elderly health and disease, combined with over 3,000 longitudinal clinical variables curated by ASPREE. Finally, We describe opportunities for statistical and machine-learning model development, harnessing the sophisticated experimental design and extensive clinical metadata curated by ASPREE.

id #89509

Proteome-wide systems genetics identifies UFMylation as a regulator of skeletal muscle function

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Maintaining or improving muscle function, in aging or disease patients, has significant potential to improve the quality of life. In fact, optimal metabolic functioning of skeletal muscle, which makes up 30-40% of an adult's body mass, is one of the best predictors of overall health. To identify potential regulators of skeletal muscle metabolism and function, we performed a proteomic analysis of gastrocnemius muscle from 73 genetically distinct inbred mouse strains and integrated the data with genomics and >300 molecular/phenotypic traits via quantitative trait loci (QTL) mapping and correlation network analysis. These data identified thousands of associations between skeletal muscle protein abundance and molecular/phenotypic traits, including plasma metabolites, lipids and cytokines; whole body measurements such as glucose/insulin sensitivity and body composition/organ weights; and muscle phenotypes such as cardiac and skeletal muscle function such as grip strength. We created an interactive web resource to explore the data at http://muscle.coffeeprot.com/. We next used this resource to target 27 genes focusing on negative associations to muscle function and performed a functional genomic screen in human micro-muscles. This screen incorporated high-throughput assessment of contractile force production and identified a series of negative regulators of muscle function including UFC1, an E2 ligase for protein UFMylation. We show that UFMylation is up-

regulated in a mouse Amyotrophic Lateral Sclerosis (ALS) model of muscle atrophy. Furthermore, *in vivo* knockdown of UFMylation increased *ex vivo* muscle function via enhanced synthesis of ribosomal and contractile proteins, and decreased protein degradation pathways including K48-linked ubiquitination. These data provide a resource to identify regulators of muscle metabolic function.

id #89222

Proteomic Landscape of Human Colorectal Liver Metastasis

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The liver is the most common metastatic site of colorectal cancer (CRC). Approximately 20% of newly diagnosed CRC will have synchronous colorectal liver metastasis (CRLM) which portends a poor prognosis, with 5-year overall survival 13%. Curative-intent surgery to resect CRLM can be effective in providing durable, long-term remission in 20-25% of patients. A smaller group of patients will have operable recurrent intrahepatic disease, while the remainder will recur with inoperable metastasis. Prognostic assessment of CRLM patient outcomes relies on clinicopathological parameters but the current models are poorly effective. To identify candidate prognostic biomarkers we mapped the proteomic landscape of 42 CRLM consisting of 17 metachronous and 25 synchronous specimens.

We obtained fresh-frozen CRLM tumour tissue from patients that underwent liver resection at Royal North Shore Hospital 2007-2017. We conducted LC-MS using a HF-X Orbitrap MS operating in DIA mode over 120 minute acquisition time. Data was searched using DIA-NN (1.8.1) in library-free mode with match between runs enabled. For peptide identification, a reverse-decoy approach was used with 1%FDR. Using this method 7450 proteins were quantified in all samples. Unsupervised hierarchical clustering of metachronous specimens indicated high inter-lesion similarity for some patients, while other patients' lesions showed discordant protein expression. Unsupervised hierarchical clustering of all specimens revealed three groups. Group 1 was enriched with extracellular matrix proteins, immune effectors and O-glycan processing proteins. Group 2 were defined by a proliferation signature of ribosome biogenesis, DNA replication and mitochondrial gene expression. Group 3 was enriched with liver metabolism related proteins. Survival analysis showed that patients with Group 2 CRLM signature experienced longer 12 month survival compared with other groups. Ongoing work includes immunohistochemistry validation and feature reduction to select the fewest effective markers for specimen classification.

id #89161

Application of shotgun proteomics techniques for characterization of proteins and peptides of tuna waste

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With the anticipated increase in human population to more than 9.5 billion by 2050, it is necessary to provide them with protein, and fish is one of the richest sources of protein available. Production of fish, both wild-caught and in aquaculture, has increased in recent years. This increase means there is more fish waste since, for example, about 36 % of tuna after processing is consider as waste or by-products. However, this by-product is rich in protein, and we are investigating ways to repurpose it and give it a second life.

Tuna trimming meal is made after food processing of tuna and is used in aquaculture. This material is a mixture of bones, skin, scales, muscles and anything else remaining. Although this material contains a lot of protein, it is not rich in some essential amino acids and the percentage of ash content is high.

In this project, we will start by performing a detailed molecular characterisation of tuna trimming meal, in terms of protein, peptides, amino acids, essential fatty acids and lipids, total carbohydrate, fat soluble vitamins and ash content. We will then investigate a range of processing techniques with the aim of improving the nutritional content. This will involve physical separation techniques, and fermentation in the presence of carbohydrate using different bacteria including *Lactobacillus*, which has been used in a similar context previously and demonstrated the capacity to change the profile of amino acids and peptides.In our initial experiments, amino acid analysis showed that 58% of the sample is protein but it only contains 2% methionine, which is very low. Similarly, the material was found to contain 0.88 % carbohydrate, which indicates that supplemental carbohydrate will be required for any fermentation experiments.We have performed a detailed proteomics characterisation of the material using SDS-PAGE to fractionate proteins and high pH reversed phase fractionation techniques to fractionate peptides, prior to nanoflow liquid chromatography – tandem mass spectrometry. The SDS-PAGE results contained 354 reproducibly identified proteins, with peptides covering the mass range from 822 to 1782 Da, including large amounts of collagens, keratins, actin, myoglobin, cytochrome c oxidase, myosin, and ATP synthase.

id #89672

Quantifying 1000 protein groups per minute of gradient using Zeno SWATH data-independent acquisition (DIA) on the ZenoTOF 7600 system

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High-throughput proteomic analysis has long been the goal of many researchers and this workflow has been shown to work well using the Evosep One HPLC system when coupled to a variety of mass spectrometry systems. Data-Independent Analysis (DIA) has become the standard method of analysis with many researchers. Zeno SWATH DIA on the ZenoTOF 7600 system gives incredible speed and sensitivity when performing these types of experiments, but also with very high quantitative precision, especially when the quantitation is performed on the MS2 transitions.

We have previously shown using a combination of the Evosep One and the ZenoTOF 7600 system that impressive numbers of proteins can be identified and quantified from standard cell lysate digests using Zeno SWATH DIA [1]. Here we present this workflow using a conventional UPLC system, the Waters M-Class at 10ml/min. The gradients used were designed to approximate the active gradient used on the Evosep One and cell lysate digests were separated on a Kinetex XB C18 150 x 0.3mm column either in direct injection or trap/elute modes.

A trap/elute method was designed using a 5-minute active gradient and 11 minutes total run time to mimic the Evosep 100SPD method. Using human cell lysates of K562, HeLa and HEK293 and a 200ng on-column load, approximately 5000 protein groups could be quantified using Zeno SWATH DIA, which is approximately 1000 protein groups per minute of active gradient. These analyses were very robust with about 90-95% of proteins being quantified with a CV <20%. These analyses were not restricted to human cell lines, as impressive data was also obtained with plasma, human urine, maize extracts and with a yeast proteome sample over 3100 protein groups could be quantified in 5 minutes.

To visualise the data obtained from DIA-NN software, we used the OneOmics suite to import the tsv output file, followed by statistical analyses to show differential protein expression. For the comparison of the three human cell lines this highlighted the reproducibility of Zeno SWATH DIA in combination with these very fast gradients.

1. Batruch, I, Le Blanc, Y, Perez, J-P and Hunter, C. "High-throughput quantitative proteomics using Zeno SWATH data-independent analysis (DIA) and the Evosep One system" SCIEX technical note, RUO-MKT-02-14799-A.

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id #89153

Species identification of a collection of worked bone artefacts from Pyrmont, Australia using mass spectrometry of bone collagen

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Zooarchaeology by Mass Spectrometry (ZooMS) is an increasingly utilised peptide mass fingerprinting (PMF) technique in archaeological science that analyses collagen 1A1 and 1A2 (COL1A1 and COL1A2, respectively) marker peptides for the genus- or species-level identification of fragmentary bones in the archaeological record. Traditionally, this analysis is performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) to identify characteristic m/z values of known marker peptides however MALDI instrumentation is becoming increasingly niche in mainstream proteomic laboratories, thus limiting the availability of this analysis. Here we present data on the application of a modified paleoproteomics approach, using nanoflow liquid chromatography - tandem mass spectrometry proteomics, to the analysis of a collection of six early colonial Australian (early to mid-19th Century CE) worked bone artefacts, believed to be mostly knife handles, excavated from a site in Pyrmont, Sydney, Australia in 2017. The historical context suggested that the bones were likely to be from sheep, cattle, whale or perhaps Australian native species. These bone handles display no distinguishable morphological features that are typically used in osteoarchaeological analyses of species; thus, a molecular approach is required for their identification. Mass spectrometric analyses were performed on a Thermo Q Exactive Orbitrap coupled with a Thermo Easy-nLC1000 liquid chromatography system. Peptide-to-spectrum matching was performed against the total curated SwissProt database supplemented with additional mammalian collagen sequences from NCBI, using the X! Tandem algorithm operating under the GPM user interface, where high-confidence positive identifications for *Bos taurus* COL1A1 and COL1A2 peptides were returned. Pairwise statistical analysis of identified peptide sequences was performed using a Fitch Matrix against a database of common domesticated species and selected Australian native mammals. This confirmed high correlation to sequences from the Bovinae subfamily, while excluding other possibilities. Taken together, these data indicate that the bone artefacts were prepared from bones of domestic cattle.

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id #89488

Conserved T cell epitopes offer protection against highly pathogenic avian influenza A/H5N1 in South Asian populations

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The influenza A virus (IAV) is well known for its antigenic drift and shift, which enables this virus to be a continuous endemic and pandemic threat. Since its first detection in 1995, the highly pathogenic avian influenza A/H5N1 has spread throughout Asia and globally and is considered as high risk for future pandemics. Current antibody-inducing vaccines that target viral surface glycoproteins (i.e. hemagglutinin (HA) and neuraminidase (NA)) can combat infection, but they often fail to protect against antigenically distinct strains of the virus. In contrast, CD8+ T cells that recognise viral peptides presented on the cell surface by class I Human Leukocyte Antigen (HLA-I) molecules mediate direct killing of virus-infected cells. Viral peptides derived from internal proteins are often highly conserved across strains, and can provide broadly cross-reactive or "universal"

immunity against different strains. In this study we examined a frequent HLA allotype associated with low morbidity and mortality rates following exposure to avian A/H5N1 influenza in South Asian populations, despite high exposure to A/H5N1-contaminated poultry. Here, we examined the HLA-A*33:03 immunopeptidome to identify A/H5N1 viral peptides recognised by CD8^{+} T cells that could be used to formulate a universal influenza vaccine.

Cells expressing HLA-A*33:03 (C1R.A*33:03) were used as antigen-presenting cells for transfection of targeted avian influenza H5N1 proteins. Using immunoaffinity purification coupled with mass spectrometry, we identified 33 potential CD8^{+} T cell epitopes from A/H5N1 proteins; including HA, polymerase basic protein 2 (PB2), matrix protein 1 (M) and nucleoprotein (NP). These candidate peptides were screened by *in vitro* stimulation of peripheral blood mononuclear cells from HLA-A*33:03-positive healthy donors to determine their immunogenicity. Strikingly, two immunogenic H5N1 epitopes (PB2$_{128}$ & NP$_{413}$) elicited strong immune reactivity and are >90% conserved among currently circulating avian and human influenza viruses. As these healthy donors have not been exposed to A/H5N1, T cell reactivity towards these two epitopes confers cross-strain protection. Moreover, single-cell profiling of NP$_{413}$-induced T cells revealed a single dominant T cell receptor clonotype. These findings can inform the development of rationally designed influenza vaccines capable of inducing cross-strain protection from severe influenza disease in HLA-A*33:03 expressing individuals.

id #89251

Shotgun proteomics to validate the differential expression of clinically relevant lncRNA-miRNA axis to reduce TMZ resistance in GBM

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Glioblastoma (GBM) is the most common form of adult-type diffuse gliomas with the aggressive capability of invading surrounding brain tissue, promoting resistance to treatment and tumour recurrence. Although the chemotherapy drug, Temozolomide (TMZ) was introduced to increase the lifespan of GBM patients by 2 months, only about 50% of GBM patients respond to TMZ and within this population, most patients develop resistance to TMZ. The development of resistance to TMZ can also be influenced by the aberrant expression of long non-coding RNAs (lncRNAs) and the expression of unmethylated O^6-methylguanine (O^6-MeG)-DNA methyltransferase (MGMT). Numerous lncRNAs have been predicted to be associated with TMZ resistance however, most of these lncRNAs are not expressed in clinical samples of GBM patients. Hence, this study bridges the pre-clinical to the clinical gap by identifying differentially expressed lncRNAs, miRNAs, mRNAs and proteins in clinical samples of primary and recurrent GBM (TCGA Firehose Legacy) which will be validated in GBM cell lines.

The differential expression of lncRNAs was determined using R programming, followed by multivariate and survival analysis to identify the lncRNAs that had the most significant impact on GBM patients' survival. The target miRNAs, mRNAs and proteins of the differentially expressed lncRNAs were identified using The LncRNA and Disease Database. To validate the expression of the target miRNAs, mRNAs and proteins in GBM cell lines, liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed. Proteomic changes were measured in GBM cell lines that were sensitive and resistant to TMZ to identify potential RNA-based markers. The selected lncRNA was then silenced or overexpressed in GBM cell lines prior to TMZ treatment to evaluate their role in reducing TMZ resistance.

Our data showed a total of 5 differentially expressed lncRNAs. LC-MS/MS proteomic profiling revealed a reduction in anti-apoptotic proteins and an increase in pro-apoptotic proteins. Knockdown of the lncRNA prior to TMZ treatment further reduced cell viability and increased the apoptotic cell population (p<0.05) when compared to the control and TMZ-only treated group.

In summary, our data suggest that lncRNAs identified from clinical datasets decrease TMZ resistance by increasing TMZ-induced cell death in GBM-resistant cell lines. Future work will focus on elucidating autophagy and ferroptosis mechanisms regulated by lncRNA to gain a comprehensive understanding of TMZ resistance mechanisms in GBM. Through this, clinically relevant lncRNAs will be identified and can be used as RNA-based markers for detecting TMZ resistance in GBM patients.

id #89191

From smartphone to mass spectrometer, a novel pipeline for food safety testing

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Publish consent withheld

id #89428

C18ORF25 is a novel exercise-regulated AMPK substrate mediating skeletal muscle function

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Exercise regulates a diverse array of phosphorylation networks thought to promote numerous health benefits and therefore hold great promise as novel therapeutic targets. Recently, we performed phosphoproteomic analysis of human skeletal muscles subject to endurance, sprint, and resistance exercise to identify canonical signalling pathways during and after exercise. This identified 5,486 phosphosites regulated during or after at least one type of exercise modality and only 420 core phosphosites common to all exercise. One of these core phosphosites was Ser-67 on the uncharacterized protein C18ORF25 which we validate as an exercise-regulated AMPK substrate. Interestingly, integration with human genome-wide association studies linked genetic variants of C18ORF25 with glycated haemoglobin and type II diabetes.

To functionally characterise C18ORF25, we generated a whole-body knockout (KO) mouse model. KO mice gained similar weight on a chow diet compared to wild-type (WT) littermates, but we observed a striking increase in adiposity and subtle decrease in lean mass. Interestingly, KO mice on a chow or high-fat diet displayed no major differences in whole body glucose tolerance or skeletal muscle insulin sensitivity as assessed by *ex vivo* insulin-stimulated glucose uptake. Also, forced treadmill exercise revealed KO mice fatigue quicker than WT mice. These data prompted us to further investigate skeletal muscle function revealing KO mice have reduced *Soleus* force production, increased fatigability and recover slower than WT siblings. Histological analysis revealed no difference in muscle fibre-type but a drastic reduction in fibre cross sectional area.

Moreover, proteomic analysis of *tibialis anterior* muscles from KO mice revealed increased extracellular matrix proteins. In contrast, loss of C18ORF25 resulted in a reduction of proteins associated with translation, pyruvate and branched-chain amino acid metabolism, NEDDylation and several mitochondrial metabolic pathways. Interestingly, the most significantly down-regulated protein in KO muscles was cAMP-dependent protein kinase catalytic subunit beta. Phosphoproteomic analysis of KO *soleus* muscles subject to *ex vivo* contraction revealed elevated phosphorylation of substrates downstream of MEK and LCK while substrates of PKA, ERK, MK2 and GSK3 displayed attenuated contraction-induced phosphorylation.

Taken together, our data suggest C18ORF25 plays a vital role in AMPK-mediated skeletal muscle adaptations to exercise and that loss of C18ORF25 attenuates several known exercise-induced signalling pathways and kinases including PKA that mediate skeletal muscle contractile function.

id #89628

Optimisation of multi-omics MALDI-MSI of lung tissue from mucopolysaccharidosis (MPS) disease murine models

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Publish consent withheld

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id #89310

Balancing the fine act of single cell proteomics

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Single cell proteomics is making strides in the field of protein mass spectrometry. Newly customised as well as automated sample preparation techniques are being used to capture and process single cells for proteomic analysis. In addition, the enhanced sensitivity and speed of new-generation mass spectrometers is making way for novel paradigms in acquisition and analysis of samples at single cell resolution. The ability to capture molecular signatures at sub-nanogram levels has been shown to be of enormous significance in studying cell differentiation, cell perturbations and systems biology, just to name a few. However, working at such low concentrations poses certain challenges in terms of establishing and optimising methods that balance the trade-off of throughput and capturing the proteome besides the intricacies of single cell sample preparation.

Using low-/ultra-low nano flow rates with single cell equivalent concentrations from commercially available HeLa digests, we identified about 350 proteins/ ~800 peptides consistently by MS2 at single cell equivalent concentrations on a Thermo Scientific[TM] Orbitrap Exploris[TM] 480 acquired using FAIMS dual CV data dependant acquisition method. As expected, using match between runs (MBR) between individual single cell equivalent acquisitions marginally increased the number of identifications to ~400 proteins/ ~1000 peptides with a majority of them being identified by a single peptide. In contrast, applying MBR using a ten-cell equivalent library (which is the most commonly used approach described in recent literature), we observed a significant increase in the number of identifications to about 1000 proteins. This approach drastically improves the conversion rate of single peptide hits to proteins identified by three or more peptides. Furthermore, we also observed an improvement in the identified proteins in the lower abundance range by an order of magnitude. Going forward, we are currently

using these optimised methods to analyse true single cell samples prepared through our in-house microfluidic sample preparation workflow.

Dissection of glycotype and protein changes following differentiation of human induced pluripotent stem cells to cardiomyocytes

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Investigating the Effect of Radiotherapy on the Immunopeptidome in Colorectal Cancer

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Treatment options for cancer have focused around the "3 pillars" of therapy: surgery, chemotherapy, and radiotherapy. A recent shift towards a 4th pillar, immunotherapy, aims to elicit an anti-cancer immune response. Cancer immunotherapy is one of the most significant breakthroughs in recent years, however, patients do not always exhibit curative responses, prompting the need to combine with other approaches. Thus, there is renewed focus surrounding the idea that radiotherapy can also stimulate T cells to recognise tumour cells, inducing an immune-mediated anti- tumour effect termed the abscopal effect.

For immunosurveillance, the human leukocyte antigen (HLA) molecules on cells present peptide antigens to T cells. The series of peptides that are displayed in the antigen binding grooves are defined as the immunopeptidome. The identification and study of these peptides is a powerful tool for identifying novel cancer antigens. Our aim is to identify a profile of the immunopeptidome pre- and post-radiotherapy in colorectal tumours.

Irradiation of a malignant colorectal cell line, SW480, halted active cellular proliferation but was sub-lethal and resulted in increased HLA class I expression. Subsequently, we investigated whether the immunopeptidome of these cells was also altered by the irradiation. Briefly, the immunopeptidomes of irradiated and non-irradiated SW480 cells were investigated by isolating peptides from purified HLA class I molecules and their identification evaluated by tandem mass spectrometry (MS/MS). This analysis revealed that irradiation alters the antigenic landscape of cancer cells. Irradiated cells demonstrated a significant increase in the total number of peptides presented, with a unique radiation-induced peptide repertoire emerging, curiously with a shift in length from 9mers towards 12mer peptides. Furthermore, there were more unique source proteins in the irradiated condition; DNA damage response dominated the pathways contributing to these new source proteins. This provides a proof-of-concept that irradiation impacts the immunopeptidome and consequently alters what is scrutinised by T cells.

Additional work included the preliminary analysis of colorectal biopsies. Building a picture of the immunopeptidome in healthy, tumorous, and irradiated tissue will provide novel insights into the immunobiology of radiotherapy which can be translated into innovative treatment options such as T cell- based immunotherapy.

PICS and TAILS N-Terminomics Characterization of Monkeypox and SARS-CoV-2 protease active sites and identification of novel Anti-viral Neanderthal Gene Products that are inactivated by SARS-CoV-2 Proteases to Circumvent Host Protection to COVID-19

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Proteases are vital components of viral protein repertoires, and antiviral inhibitor drugs have been game-changers, *e.g.*, HIV AIDS, and hepatitis C. Hence, it is incredibly important to predict the outcome of therapeutic inhibition of the proteases in zoonotic infections and diseases, including COVID-19 and monkeypox. This can only come from understanding the full extent of protein cut sites and proteolysis of host cellular substrates by the viral proteases, yet most studies cherry-pick targets to biochemically screen, and few employ highly validated proteomics analyses targeted to substrate discovery. Further, all investigations of SARS CoV-2 3CLpro "main protease" have focused on the non-prime (P) side to develop anti-viral drugs, neglecting the potential of the prime (P') side to improve potency. We describe the full cleavage site specificity from P6 to P'6 for SARS-CoV-2 3CL^pro and both the cysteine and metalloproteases of monkeypox virus using __P__roteomic __I__dentification of __C__leavage __S__ubstrates (PICS) (Nature Biotechnology). For SARS CoV-2 main protease, 3CL^pro we also performed 50,000 molecular simulations per peptide in complex with the protease to model 10 of the best fitting sequences to unveil structural determinants of 3CL^pro-substrate interaction. Using three proteome peptide libraries (generated by trypsin, GluC and LysargiNase), we determined the preferred cleavage motif of the three proteases from SARS-C0V-2 and monkeypox. Using a

total of 816 cleavage sites from CoV-2, we developed soluble and high-efficiency FRET probes to monitor 3CL[pro] activity. In this analysis, we uncovered a unique target that is a Neanderthal gene product that showed the highest protection from viral infections, yet was found to be a novel SARS-CoV-2 protease substrate. Employing SARDS-CoV-2 infection and transfections of noncleavable vs. cleaved analogues, we show the essential role of this protein and decipher how SARS-CoV-2 defeats its function to lead to worse infection and COVID-19. This analysis has broad implications for extending the chemical envelope of possible chemical routes to improve the treatment of COVID-19, to develop new drug scaffolds prepositioned to adapt to emerging zoonotic Coronavirus spillover to human populations for pandemic preparedness in the future, and, at the same time providing new biological insights into how the virus evades the host immune response and drives replication. Thus, TAILS and PICS are potent terminomics approaches to decipher the pathogenesis of COVID-19, Monkeypox and other emerging viral diseases and to improve anti-viral drug design to treat viral infections now and in the future.

id #89756

Inhibition of ERAP1 in cancer unmasks an alternative subset of tumour epitopes recognised by cytotoxic human CD8^{+} T cells

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The antigen processing machinery of a cell shapes the repertoire of peptides presented for immunosurveillance on the cell surface. Human leukocyte antigen class I (HLA-I) molecules present peptides that are recognised by both CD8^{+} T cells and NK cells, and these complexes play a vital role in the recognition and eradication of transformed cancer cells. During direct presentation, endoplasmic reticulum aminopeptidases (ERAPs) can further process peptides translocated into the ER following intracellular proteasomal degradation, thereby editing the final pool of ligands able to compete for binding to HLA-I molecules. Hence, modulation of ERAP processing presents an opportunity to profoundly alter the immune landscape of cancer. Here, we developed two highly potent and selective allosteric inhibitors of ERAP1, and showed that pharmacological inhibition of multiple ERAP1 haplotypes across several cancers is able to unmask an alternative suite of tumour-associated peptides that are presented by a diverse array of HLA-I molecules. Crucially, several epitopes either significantly upregulated or uniquely presented following ERAP1 inhibition were recognised by pre-existing CD8^{+} T cell populations in multiple healthy human donors, demonstrating that pharmacological inhibition of ERAP1 may be able to both augment anti-tumour immunity and subvert tumour immune evasion mechanisms by unveiling an alternative subset of epitopes with the capacity to prime novel cytotoxic T cell responses.

id #89482

Exploring the phospho-immunopeptidome in haematological malignancy and their specific T cell receptors in cancer precision medicine

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Haematological malignancy (HM) is an umbrella term used for neoplasms originating in haematopoietic and lymphoid tissues. Treatment guidelines for HM includes induction therapy or allogeneic hematopoietic cell transplantation (allo-HCT). Although allo-HCT is considered an optimal approach, transplant-related mortality is seen in significant numbers in HM patients. Hence alternative forms of treatment must be explored. Dysregulated phosphorylation at serine, threonine and tyrosine residues is a hallmark of neoplastic transformations. Phosphorylation at these residues is retained even after proteins are processed by human leukocyte antigen (HLA) class I machinery and presented as peptides on cell surface of cancer cells (known as the immunopeptidome). These neoantigens can be targeted for immunotherapies such as T cell receptor (TCR) based vaccines, chimeric antigenic receptor T cells and bispecific antibodies. In this study we have used mass spectrometry to identify the phospho-immunopeptidome in HM cell lines and patient bone marrow samples. The immunogenicity of some of the phosphopeptide was assessed in patient peripheral blood mononuclear cells.

A total of 81,027 HLA class I peptides were identified from 3 cell lines and 4 clinical bone marrow aspirates. A total of 1,733 phosphopeptides including rare tyrosine phosphorylated (restricted to HLA A2) were detected. The study reports for the first time of identifying TCRs specific for phosphopeptides presented by HLA-A2 and -B07 in HM patients.

Since phosphorylation is driving force across several tumour malignancies, identification of phosphopeptide specific TCRs will aid in development of novel immunotherapeutic approaches.

id #89300

The ProtoemeRiver pipeline facilitates reproducible and sharable differential abundance and pathways analyses of proteomics and phosphoproteomics datasets

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Identifying phosphorylation sites and how they change in abundance under different environmental conditions are important for elucidating the role of signal regulations in cellular processes. ProteomeRiver is a novel pipeline that facilitates the analysis of differential abundance of proteins and their phosphorylation events. The pipeline enables the batch analysis of many pairwise treatment versus control comparisons and subsequent pathways overrepresentation analysis. To enable differential abundance analysis of mono- and multi-phosphorylation events, ProteomeRiver incorporates missing values imputation (PhosR*, 1), removal of unwanted variation (ruv*, 2), normalization between samples and linear models (limma*,3), kinase-substrate enrichment (KinSwingR*, 4), and pathways analysis (clusterProfiler*, 5). The changes in the abundance of phosphorylation events were also normalized by the changes in host-protein abundance using custom scripts. This pipeline uses modular components, which allows the modules to be substituted and/or extended with novel tools as they become available. This pipeline also uses a small set of configuration files and scripts to store all instructions necessary for data analysis, which could be shared publicly on code repositories to support reproducibility. We demonstrate the pipeline through the re-analysis of a published dataset of synapses proteome and phosphoproteome during homeostatic up- and down-scaling (6). The use of ProteomeRiver resulted in the identification of pathways and upstream kinases associated with homeostatic up- and down-scaling, with the number and types of pathways identified comparable to those from the original publication. ProteomeRiver enables all steps necessary for the analysis to be easily shared with other researchers and for them to independently reproduce the results. The pipeline and step-by-step tutorial will be available as a R package via https://bitbucket.org/cmri-bioinformatics/proteomeriver. (*) denotes R or Bioconductor packages.

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id #89256

High sensitivity immunopeptidomics on the timsTOF SCP mass spectrometer

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Immunopeptidomics aims to resolve the composition and dynamic changes of endogenous peptides presented by major histocompatibility complex (MHC) proteins. This peptide display-recognition system enables the elimination of infected, cancerous, stressed, and damaged cells by cell-mediated immunity, alongside supporting antibody production and innate immune responses. The identification of disease and tumour-related HLA presented peptides in cell lines and biopsies will progress our understanding of disease pathogenesis, immune responses and aid the preparation of targeted vaccines and immunotherapies.

Existing proteomic methodologies are adapted to target tryptic peptides and are often not optimal for immunopeptidomic analyses. For example, class I peptides are present at exceptionally high dynamic ranges, they are shorter (8-14 amino acids) than tryptic peptides, and have a variable, often hydrophobic, C-termini. These characteristics result in a high number of singly charged ion species and fragmentation spectra of lower quality due to the missing dominant y-ion series observed for tryptic peptides with basic C-termini.

Here, we optimise trapped ion mobility spectrometry (TIMS) in the timsTOF SCP mass spectrometer for immunopeptidomics analysis. TIMS allows for improved signal to noise, better DDA sampling by using mobility windows, and 5-fold higher transmission of ions which boosts the identification rates particularly in low-abundant samples.

We validate our methods with a synthetic standard containing a predefined diverse repertoire of 2000 HLA peptides, observing allele dependent peptide sequencing bias. Next, we demonstrate this application on an immunopeptidomic sample generated from a leukemic T-cell lymphoblast cell line, determining sensitivity related to cell count and peptide load. Finally, we perform an intra-laboratory comparison to a HF-X Orbitrap type mass spectrometer. We observe striking improvements in lower limit of detection in the timsTOF SCP, but find that instruments are complementary when sample load is not a limiting factor.

id #89158

Glycosylation of the SARS-CoV-2 Gamma spike protein

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) - the cause of the ongoing COVID-19 pandemic - has the capacity to generate variants with major antigenic changes in the surface exposed spike glycoprotein. The emergence of SARS-CoV-2 viral variants threatens current anti-viral and preventative strategies, including monoclonal antibodies and vaccines. The trimeric spike protein of SARS-CoV-2 mediates binding to and fusion with host cells and is covered with a

"glycan shield" of ~66 N-glycosylation sites per trimer. Glycosylation is required for efficient folding and maturation of the spike proteins, and the specific glycan structures on the viral proteins can control their interactions and thereby the specificity of viral infectivity and immune evasion. To gain a better understanding of the role glycosylation plays in SARS-CoV-2 we investigated the glycosylation profile of a recently emerged Gamma variant which has mutations that introduce two new *N*-glycosylation sequons. We expressed and purified the original Wuhan spike, the Gamma variant and a combination of variants from CHO cells, and performed glycoproteomic analysis, which showed changes to their site-specific *N*-glycosylation occupancy and structural heterogeneity. We observed site swapping with the introduction of a downstream *N*-linked sequon at position 20 in the spike protein of SARS-CoV-2. Our work also revealed that sequence changes in the spike proteins can dramatically alter the glycosylation profile of the protein. This work highlights the difference in glycosylation of the spike protein between variants which can be used to inform therapeutic strategies.

id #89077

How to make green, sustainable and on-demand Nitrogen for your Mass Spectrometer and Proteomics research from Air

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Nitrogen, composing 78 % of the earth's atmosphere, is the 4th most abundant element in the human body and equally essential in scientific research for the ultimate performance of a mass spectrometer.

The classical method of purifying N_2 is by cryogenic separation; Air is cooled to – 190 $^\circ$ C and O2 and N2 separated in liquid form. This is then transported to required site for use, typically through evaporation, as N2 in its gaseous form. This leads to wastage though 'Boil off' as gas travels from plant room to laboratory of 0.2 % to 5 % per day, in addition to transport, delivery and safety costs.

Nitrogen cylinders can be filled from off-site N2 purification and booster compressor. Cylinders can be high risk due to high pressure the gas is storage under and weight to move. A typical Mass spectrometer will use up to 1 cylinder per day, equating to 2000 G-size cylinders a year - leading to high off-site energy production and transport carbon costs.

This presentation details how N_2 gas can be purified on site & on demand at desired flows, pressure and purity specifically tailored to your proteomics research and Mass Spectrometer of choice.

N2 generators are small (fit under a standard Laboratory bench),

Quiet (think hum of an air conditioner),

Use < 10 amp power (greener for the environment) and

Only requires 1 x delivery (decarbonisation due to less truck deliveries).

There is no storage of gas a high pressure or volumes, hence safer and easier for laboratory staff to use 24/7.

id #89393

Multiplexed interrogation of selective inhibitors of fatty acid-binding protein 4 by native mass spectrometry

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Fatty-acid binding proteins (FABPs) are a family of intracellular lipid chaperones that regulate the uptake and intracellular distribution of lipophilic ligands such as fatty acids.[1] There are 10 isoforms of FABPs and although these isoforms show only around 20-70% sequence similarity, they share a highly-conserved tertiary structure.[2] Several known drugs bind to FABPs, but they show poor isoform selectivity and often show similar binding specificity to nuclear hormone receptors, thereby limiting their utility in selectively targeting FABPs. Therefore, development of compounds that display significant selectivity for various FABP isoforms will be valuable tools to dissect the cellular functions of FABPs.

Multiple approaches for the characterisation of protein-ligand interactions are available in fragment-based drug discovery but many are often associated with drawbacks, especially when employed for target selectivity studies. Typically, current approaches for target selectivity involve generating ligand affinity data for individual proteins of interest, which is often both time-consuming and resource-intensive. Native mass spectrometry (nMS) is an emerging technique with the capability for addressing such challenges, enabling multiplexed analysis of heterogenous mixtures while requiring only picomole quantities of analytes. Whilst there is limited information of nMS employed for the investigation of ligand selectivity against multiple protein

targets, through a similar principle, nMS has shown potential as a target identification approach, with a recent study successfully probing the protein partner of a single ligand from a protein mixture.[3]

We have developed a nMS workflow using Orbitrap detection for the interrogation of target selectivity of FABP inhibitors. Exploiting the multiplexed nature of the technique, we employed nMS for simultaneous evaluation of the selectivity of a single ligand for five different FABP isoforms. We found good agreement of selectivity profiles generated by nMS with those derived from nuclear magnetic resonance spectroscopy and isothermal titration calorimetry. This study demonstrates the capability of nMS for rapid, multiplexed screening to simultaneously identify hits with both favourable binding affinity and selectivity profiles, thus highlighting the potential for screening campaigns to be accelerated.

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id #89090

C3, a collection of data management, visualization and exploration web applications for proteomics data analysis

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Visualization of differential expression mass spectrometry data is normally undertaken by a mass spectrometry expert using software suites such as Perseus or R that require computer installation and significant expertise to use. This makes it difficult for researchers without such software or expertise to obtain the visualizations they want on their own. Additionally, it makes it difficult for the person with the skills, software and expertise to share the data with non-expert collaborators. To circumnavigate this, we created a collection of free to use, open-source, and web-based tools that can perform data management, data visualization, and data exploration of mass spectrometry-based proteomics data called "C3". C3 comprises of "Celsus", "Curtain" (http://curtain.proteo.info) and "CurtainPTM" (http://curtainptm.proteo.info).

Across the 3 applications, these tools share various common data visualizations such as volcano, profile, bar and network charts. Celsus puts the focus on being a single repository for storing all of your analytical output from multiple projects where you can also quickly search for any proteins of interest across stored results. Being a web-based application, only one instance is needed for your whole laboratory's data to be accessed within an internal network or across the internet.

Curtain and CurtainPTM, however, have a different focus toward sharing a particular experiment's results along with visual customization for certain hits or proteins of interest. Here, the application also aggregates additional information such as summary of known biological function, domain structure, AlphaFold structure and characterized interactors of each selected protein. The users can search for an individual protein or a collection of proteins by providing the gene names, UniProt accession ids, or using our list of built-in protein collections associated with various themes such as Parkinson's Disease, Alzheimer's Disease, kinases, phosphatases, mitochondrial proteins, glycosylation proteins. In CurtainPTM (PTM specific version), it also allows phospho-proteomic and other post-translational proteomic data to be easily analysed and visualised in a similar manner. Identified phosphorylation sites and how these vary between experimental samples can be plotted and compared with phosphorylation site data reported within the PhosphoSitePlus database. The data, as well as the proteins or PTMs that the user chose to highlight, can be sent onto anyone along with any user-determined visualization customization through a unique web link that can be saved and shared.

id #89418

Accelerating DIA studies with fast microflow LC and Zeno SWATH acquisition

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As the field of quantitative proteomics continues to evolve, larger biological cohorts are being studied, often using precious samples obtained from biobanks or other difficult-to-obtain sources. This creates 2 workflow requirements: the need to acquire quantitative data on the digested samples faster and the need to use smaller amounts of sample. For these types of studies, data-independent acquisition (DIA) continues to grow as the workflow of choice for reproducible quantitative analysis of large numbers of proteins from a proteomic sample.

As such, new workflows and software tools have emerged. Previously, the combination of fast microflow chromatography and SWATH DIA enabled large numbers of proteins to be quantified from complex proteomics samples at very high rates, up to 100 samples per day.[1] Zeno MS/MS on the ZenoTOF 7600 system provides ~5- to 6-fold increase in peptide MS/MS sensitivity and can be used in MRM[HR], DDA and DIA workflows.[2,3,4] Also, multiple powerful algorithms have emerged that have enabled more proteins to be identified and quantified from DIA data, such as DIA-NN software.

Here, the improvements in proteins identified and quantified using microflow SWATH DIA coupled with Zeno MS/MS is described. Four different gradient lengths (5, 10, 20 and 45 minutes) were tested to cover a range of application needs. The library-free approach to processing DIA data (using in silico generated spectral libraries) was also evaluated vs. the traditional shotgun proteomics approach with Zeno DDA. Other workflow comparisons were performed to benchmark the workflows. DIA data were processed with DIA-NN software and DDA data were processed with ProteinPilot app in OneOmics suite.

1.   1. Accelerating SWATH acquisition for protein quantitation – up to 100 samples per day. SCIEX technical note, RUO-MKT-02-8432-A.
2.   2. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
3.   3. Large-scale, targeted, peptide quantification of 804 peptides with high reproducibility, using Zeno MS/MS. SCIEX technical note, RUO-MKT-02-13346-A.
4.   4. Zeno MS/MS with microflow chromatography powers the Zeno SWATH DIA workflow to quantify more proteins. SCIEX technical note, RUO-MKT-02-14668-A.

id #89420

A novel fragmentation technology allows for in-depth glycopeptide characterization in glycoproteins

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Improving the depth of characterization of glycoprotein is essential in the field of biotherapeutics and mAbs analysis. The newly developed electron activated dissociation (EAD) approach allows to tune electron energy to produce different fragmentation patterns of the electron-based dissociation (ExD) family.

In this study monoclonal antibodies adalimumab, rituximab as well as an antibody-drug-conjugated sample (ado- trastuzumab emtansine) were digested into peptides and acquired on the ZenoTOF 7600 using fast data-dependent acquisition (DDA) combined with EAD alternative fragmentation.

This novel fragmentation technology resulted in rich fragmentation with predominantly peptide backbone fragments leaving the glycan intact on the glycosylation site, enabling localization and providing a higher level of structural information for glycopeptide   characterization.
Thanks to the Zeno trap the detection level of the diagnostic fragments was increased by 5 to 10-fold

The data presented here demonstrate the advantage for glycoproteins using this novel fragmentation cell over traditional collision-induced dissociation (CID) used for peptide mapping, which does not allow for consistent identification and localization of glycans on peptides. Combining increased detection of fragments using a mechanism for increased duty cycle on a Q-TOF instrument (the Zeno trap) enables higher confidence in data assignment, making the Zeno EAD combination ideal for in-depth analysis   of   glycopeptides.
We are also demonstrating the power of the SCIEX OS software and automated data interpretation with the new Biologics Explorer software offering a streamlined characterization workflow.

id #89620

Automated benchmarking allows rapid development and fast confidence

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Protein characterization, including peptide mapping and multi-attribute monitoring using LC-MS/MS methods are widely adopted techniques in both academic and industrial drug development settings. Peptide Mapping workflows are computationally complex, involving many algorithmic steps, usually requiring manual input and decision making from the end user and therefore extra effort to guarantee reproducibility of the results. While automated workflows are increasing in popularity, there is a need to make sure that ease of use doesn't come at the expense of quality and transparency. Here, we present a standardised approach to benchmarking and quality control of protein characterisation workflows.

The Mass Dynamics Peptide Mapping service is a fully automated, cloud-based LFQ-DDA workflow that presents identified and quantified peptide spectral matches via an easy to use web interface. To guarantee the ongoing accuracy and appropriateness of all algorithmic steps including Feature Detection, Database Search, Target-Decoy Scoring etc.., we developed automatic and interactive quality control reports generated alongside experiment results. Building on this work, we developed an automated benchmarking service using publicly available data with known concentrations of spiked proteins that can validate and evaluate each new release of the workflow.

The quality control report provides plots and summary statistics for each step in the workflow. This includes for example the number of features detected in each sample; the cross correlation distribution between target and decoy after peptide scoring and the mass over charge error distribution after performing MS1 recalibration. Detailed documentation of the interpretation and expected behavior is provided for each reported statistics and this will allow quick comparison between experiments and an increased understanding of how to detect outlier samples and characterize the quality of an experiment.

Benchmarks using the ground truth data are run with every new release of the workflow and are available to the user for inspection. Therefore, this automated benchmarking setup allows the user to maintain trust and reliability in the service as well as it allows a robust and efficient development and testing of the workflow by providing real-time results using ground truth data.

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id #89366

Quantitative Tissue analysis reveal Adenylate kinase 2 protein signatures: therapeutic target for meningioma

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Arising from the arachnoid villi cells, meningioma constitutes 35% of all the primary brain tumors. According to WHO, meningiomas can be classified into 3 grades i.e. WHO grade I (typical), WHO grade II (atypical), WHO grade III (anaplastic), further dividing them into 15 subtypes. Based solely on histology, nine of these subtypes comes under WHO grade I and three each in grade II and grade III. Though benign, 80% meningiomas can be treated by resection and chemotherapy thereafter. However, 20% recur and need further surgery, radiotherapy or another round of chemotherapy. Moreover, comorbidities like neurological and cognitive disorders can be caused by relatively indolent meningiomas. Impede cognition, in grade I patients further diminishes the life surviving conditions. Though, meningiomas are the most common intracranial tumors, occurrence of multiple meningioma (10% of the affected population) present another set of complications to deal with. Biomarkers as 'targets' may provide a better treatment and prognostic factors for these patients and their follow up routines. Proteome studies that aim to decipher novel insights in the tumors provide a vast reservoir for likely molecular targets. However, non-availability of in depth proteomic re-searches in this field renders the treatment dubious. Herein, we present a proteome analysis of meningioma patients aimed to decipher the role of selected functional proteins which may serve to be target for therapies. The study presented here dwell on the proteins like adenylate kinase 2 (AK2), collagen type 1 alpha 1 (COL1A1)), plasminogen (PLG), found via mass spectrometry analysis. Simultaneously, cell sorting by FACS was used to support the mass spec findings. Furthermore, these 'protein markers' might be targeted to identify another panel of proteins that can be screened in a large patient cohort using targeted proteomics.

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id #89547

Modified mRNA encoding Myc and Cyclin T1 induces cardiac regeneration following myocardial infarction

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Following severe myocardial assault, such as myocardial infarction (MI), cardiomyocytes perish and are never replaced. The permanent loss of these contractile cells leads to deteriorated heart function and ultimately heart failure. Hence, there has been intensive interest in uncovering the molecular aetiology that control myocyte proliferative capacity to augment adult cardiomyocyte turnover for heart regeneration. We recently showed that transgenic expression of the pro-proliferative transcription factor Myc and positive transcription elongation factor (P-TEFb) complex elements, namely Cyclin T1, drives substantial adult cardiomyocyte proliferation. However, the sustained whole heart expression leads to functional decline and death over 4 days.

In this current study, we hypothesised that careful titration of the dose and transient timing of Myc and Cyclin T1 may be required to sustain cardiomyocyte proliferation and cardiac regeneration *in vivo*. Human cardiac tissues derived from pluripotent stem cells were exposed to modified mRNA (modRNA) containing Myc and Cyclin T1 sequences. The proteomic response was monitored at 1, 4, 18 and 48hrs post-transfection to identify the pathways stimulated through ectopic expression of these mitogens. Whilst the protein abundance of Myc and CyclinT1 is transient and depleted within 48hrs, the downstream proliferative and associated pathways are activated at the later time points. Cell counts and microscopy confirmed a cardiomyocyte cell growth signature at 48hrs. Using a mouse MI model, we then tested the regenerative capacity of transient Myc and CyclinT1 through intramyocardial modRNA injection following traumatic injury. Alike the stem cell model, transient expression was observed, with DNA synthesis and mitosis assays confirming proliferative effects within 24hrs. At 28 days post-MI, a demonstrable improvement to cardiac function can be observed by increased ejection fraction and left ventricle size due to Myc-CyclinT1 expression compared to control mice.

Collectively, these findings indicate a single transient dose of Myc-CyclinT1 is sufficient to induce cardiomyocyte proliferation and combats deleterious effects of MI. The clinical potential for modRNAs has recently been demonstrated where combination with refined modRNA delivery platforms for heart-specific targeting enable the potential for Myc-CyclinT1 modRNA as an effective therapeutic.

id #89419

Unravelling phenotypic changes in gas-fermenting acetogen Clostridium autoethanogenum through proteomics

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Concerns over high greenhouse gases emissions from transportation have made us consider lower-carbon fuels. An alternative to petroleum-based fuels is engineered microorganisms that use renewable carbon sources to produce biofuels. Acetogens represent an attractive platform for production of biofuels due to their ability to metabolise carbon oxides from waste gases into high-value compounds such as acetate, ethanol and 2,3-butanediol through the Wood-Ljungdahl pathway. Here, we aim to develop a recombinant strain of the model acetogen *Clostridium autoethanogenum* to produce isobutanol, a next-generation biofuel that can be used as jet fuel and has properties that closely resemble to gasoline, including high energy density and calorific value as well as low volatility and miscibility with water. Fist, we designed an isobutanol recombinant pathway derived from pyruvate including a combinatorial assembly of enzymes from several organisms. To identify the optimal chassis strain to carry this pathway, we studied two *C. autoethanogenum* strains exhibiting different phenotypes and quantified their proteome aiming to understand the regulatory networks and genotype-phenotype relationships that could potentially lead to improved isobutanol production. Using adaptative laboratory evolution, these two strains have been adapted to grow in autotrophically solely on CO, CO_2 and H_2. One of these strains, hereafter named ALE4, shows a higher production of 2,3-butanediol, one of the secondary products from the Wood-Ljungdahl pathway. The other strain, named EvoBrini, grows more robustly, has increased specific gas uptakes and lower 2,3-butanediol production. Proteome quantification from continuous cultures of both strains at steady-state elucidated key differences in protein expression leading to these phenotypes. Proteins involved in production of 2,3-butanediol from pyruvate and Nfn transhydrogenase complex involved in generation of redox cofactors, were overexpressed in the ALE4 strain. In EvoBrini, we identified overexpression of proteins from the ethanol metabolism and biomass production. Insights into the proteome of these strains provides a better understanding of metabolic networks that could be used for improved isobutanol production.

id #89443

Utilizing CID and EAD fragmentation for global lipid profiling of human and rat plasma

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Lipidomics utilizing higher throughput methodologies typically result in a significant loss of annotated species, as either the chromatographic resolution is reduced and/or the MS/MS acquisition rate of the mass spectrometer is insufficient for the increased elution concurrency. In addition, the strategy for determining in-depth structural information can involve multiple injections and methodologies.

This work is based on a data-dependent workflow that fully leverages a hybrid collision cell containing the Zeno trap (Figure1) and electron activated dissociation (EAD) cell. Electron kinetic energies can be precisely tuned from 0-25 eV without chemical transfer reagents. The ability of the EAD cell to contain a high density of electrons allows for rapid reaction rates that keep up with fast chromatographic separations.

Previous work demonstrated the complete structural characterization of lipids using EAD MS/MS on the ZenoTOF 7600 system.[2] Here, the impact of the higher sensitivity Zeno MS/MS on the detection of lipids from extracted plasma samples was explored using a single injection, data-dependent acquisition (DDA) workflow. Chromatographic separations were significantly accelerated and the impact on identification rates was evaluated. The data were generated using Zeno MS/MS combined with EAD fragmentation to compare to the CID data.

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id #89684

Genome to phenome: exploiting multi-omics and deep learning strategies to decipher importance of protein isoforms in animal health and behavior

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Alternative splicing is a widespread capability of mammalian genomes to increase phenotypic diversity from a limited set of genes. The process results in transcript and protein complexity, and has direct implications in health, disease, behavior, and translation of animal models to therapies. Apart from being a subject to natural selection, animal genomes may also be under pressure of selective breeding, and it is paramount to understand how this genetic selection leads to a phenotype. We postulate that protein isoforms play an important role in this process and show how we leverage the advancements of long-read RNA sequencing (Iso-Seq) and deep learning neural network tools to deconvolute SWATH-MS fingerprints of clinical samples and generate isoform-resolved maps of brain and heart proteomes in animal models of behavior and myocardial infarction.

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id #89269

An ACF Multicentre Testing Initiative: Exposing the Limitations of Shotgun Proteomics

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The Australasian Core Facilities (ACF) group was established in 2019 to discuss challenges, opportunities and best practices in core facility management and mass spectrometric applications. In line with these goals, the ACF group has conducted during 2022 their first Multicentre Testing Initiative, which was aimed to (i) examine reproducibility across Australasian mass spectrometric core facilities and (ii) to identify optimal strategies and workflows – but also potential pitfalls and issues – in data-dependent acquisition (DDA) mass spectrometry, which is commonly referred to as shotgun proteomics.

To that end, two tryptic peptide samples of defined composition and differences were distributed to 21 participating mass spectrometric core facilities to be acquired in technical triplicates on any LC-MS/MS system(s) operated in DDA mode. Any acquisition method or workflow was admissible provided that (i) the linear separation gradient did not exceed 120 min, (ii) the samples were not further fractionated and (iii) less than 2 ug were injected per replicate. Following these guidelines, the ACF group has received a total 66 datasets acquired on a multitude of mass spectrometers and LC systems using a variety of parameters and settings. Upon receipt, all data were de-identified by ACF's data custodian, and forwarded to MassDynamics to be comparatively analysed and visualised using their standardised, automated pipelines.

In this presentation we will comprehensively describe the findings of this first Multicentre Testing Initiative. We will furthermore use these benchmarking datasets to expose significant limitations of shotgun proteomic approaches, but also provide advice on seemingly optimal DDA strategies and workflows.

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id #89437

Capturing and Characterising Wild Yeast for Beer Brewing

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Beer is typically made using fermentation with *Saccharomyces cerevisiae* or *Saccharomyces pastorianus*, domesticated brewing yeasts. Historically, wild, non-*Saccharomyces* yeasts have also been frequently used in mixed culture fermentations to provide interesting and unique flavours to beer. However, brewing using mixed cultures or by spontaneous fermentation makes reproducing flavours and beer styles extremely difficult. Here, we describe a pipeline from collection of native wild yeast from plant material to the characterisation and commercial scale production of beer using wild yeast. We isolated 23 wild yeast strains, performed fermentation assays and measured ethanol production. We used growth assays, proteomics, metabolomics, and genomics to understand the sugar and amino acid utilisation profiles of two candidate production strains of wild *Torulaspora* compared to commonly used craft beer brewing yeast US05, and a poorly performing wild strain of the same genus. We then investigated media composition and culture condition parameters that could affect growth, and modifications that could be made at a commercial scale to promote effective fermentation and product quality.

id #89319

Burkholderia O-linked glycosylation systems possess buffering capacity limiting the impact of perturbations in oligosaccharyltransferase levels on the glycoproteome and proteome.

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O-linked protein glycosylation is a highly conserved process across the Burkholderia genus and mediated by the oligosaccharyltransferase PglL. While our understanding of Burkholderia glycoproteomes has dramatically improved in recent years (1) little is known about the dynamics of bacterial glycosylation and how Burkholderia species respond to modulations in glycosylation levels. Utilising a Rhamnose inducible CRISPRi based system (2) we explore the impact of O-linked glycosylation silencing across four species of Burkholderia (*B. cenocepacia* K56-2, *B. diffusa* MSMB375; *B. multivorans* ATCC17616 and *B. thailandensis* E264). Across these species proteomic analysis reveals CRISPRi allows the inducible silencing of PglL levels, yet find this does not diminish glycosylation capacity in a proportional manner. Using glycoproteomics we find that even with the reduction of PglL to below detectable levels O-linked glycosylation persists across all Burkholderia species. Comparing *B. cenocepacia* ΔpglL to glycosylation silenced strains of *B. cenocepacia* at both low (0.1%) and high (1%) concentrations of Rhamnose reveals that while glycosylation can be inhibited by nearly 90% the proteome and phenotypes associated with the loss of glycosylation, such as motility, are not impacted. Importantly this work also demonstrates that high levels of Rhamnose induction can lead to extensive impacts on Burkholderia proteomes which, without appropriate controls, mask the impacts specifically driven by CRISPRi guides. Combined this work reveals the O-linked glycosylation systems in Burkholderia species appear buffered from modulations in PglL levels and that Burkholderia proteomes appear tolerant to fluctuations in glycosylation capacity.

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id #89498

Mass spectrometry method development and evaluation for host cell protein characterization from clarified cell culture fluid of biopharmaceutical processes

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Biopharmaceutical protein products have revolutionized treatment of diseases and are one of the fastest growing segments of the pharmaceutical industry. The production and purification of biopharmaceutical proteins using cellular expression systems is challenging, due to the heterogeneity of the product and complexity of parameters influencing the process. Close monitoring of Critical Quality attributes (CQAs), - attributes of the product itself or impurities that are introduced through the process known to influence efficacy, safety or stability of the final product, - is necessary. Host cell proteins (HCPs), proteins introduced by the expression system, are especially challenging to monitor and to deplete trough purification, due to a wide range of physicochemical properties like molecular weight or isoelectric points, which can often be similar to the properties of the product. The industrial standard to monitor HCP are enzyme-linked immunosorbent assays (ELISA). These assays are targeted to specific proteins of interest and only detect immunoreactive HCP species. In contrast mass spectrometry (MS) is a non-targeted approach and allows unbiased identification and quantification of HCPs population down to single HCPs. Monitoring HCP populations within the bioreactor would increase the understanding on the influence of processing conditions on their distribution and quantity, especially regarding specific HCPs that have been identified as critical for product quality and safety. Here, we present method development to monitor and relatively quantify HCP directly from clarified cell culture fluid (CCCF) samples using a bottom-up MS approach. To optimise the method, we investigated the influence of matrix interference and

influence of different sample preparation protocols, pre-treatment, liquid chromatography and MS parameters, MS acquisition techniques and the use of different software tools for data analysis. Methods and protocols were evaluated for robustness, sensitivity, ease of use, time requirements and transferability for use in an industrial environment. To mirror the highest possible complexity and the effect of high abundant proteins compared to the HCPs, the method development was carried out using CCCF samples obtained from a hybridoma cell line, which contains foetal calf serum in the culture media. The optimized method will be used to characterise and relatively quantify HCP from industrial CCCF samples over a time course in a timely manner and should be easily transferable to other applications.

id #89349

Unravelling the glycosylation profile of the oseltamivir-resistant NA protein in the A(H1N1)pdm09 strain

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Influenza A virus has been a constant threat to human population causing four major pandemics. In 2009 during a pandemic, a novel H1N1 viral strain emerged (H1N1pdm09), demonstrating resistance to the antiviral drug oseltamivir due to a single amino acid substitution H275Y in neuraminidase (NA). This resistance came at a small cost of the viral fitness, yet secondary permissive mutations such as V241I/N369K were reported to restore NA's activity to the levels of that of in the wild-type virus (1). NA is a homotetramer glycoprotein consisting of a catalytic head domain with four active sites and a stalk region that anchors NA to the virion viral membrane. Both domains contain a number of glycosylation sites that have been reported to significantly affect the pathogenicity (2), the efficient incorporation and replication of the virus in host cells, (3) as well as the structural stability of NA itself (4). However, how the glycosylation sites were affected in the new H1N1pdm09 strain remains elusive. Considering the central role of NA in maintaining influenza's transmissibility together with the evidence suggesting glycosylation alternations could implicate with NA's biological activity (2), we aim to characterise the glycosylation profile of the NA H275Y and to assess whether permissive mutations induce any changes. Here, we reproduced the mutations in the NA (A/H1N1/Auckland/2009) via site-directed mutagenesis PCR and expressed the recombinant soluble NAs (rNA) in HEK293T cells. Using the Waters Select Series cyclic ion mobility mass spectrometer and the SCIEX ZenoTOF mass spectromerter we successfully characterised the glycosylation nuances between the variable rNAs under CID and EAD fragmentation modes. Taking into consideration the accumulative evidence that constitute NA an important target for the development of next-generation flu vaccines (5), the results of this study highlight exciting future therapeutic avenues.

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id #89453

Insights into the Biological Function of Algae using Glycomic and Proteomic Approaches

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Publish consent withheld

id #89592

Mass spectrometry-based thermostability measurements of virus-derived MHC peptides correlate with immunogenicity

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The major histocompatibility complex (MHC) encodes molecules that bind and present peptides on the surface of cells to stimulate T-cell responses. Although the stability of peptide-bound MHC (pMHC) complexes can influence their immunogenicity, peptidome-wide analysis of pMHC stability has only recently been achieved (1). Here, we refined a wide-scale immunopeptidomics workflow to improve accurate thermostability profiling of thousands of individual pMHC complexes and

then assessed the extent that thermostability correlates with viral-pMHC immunogenicity. We utilized a C57/BL6 mouse DC2.4 cell line (MHC class I H2-Db/Kb) to assess the thermostability of a set of vaccinia virus-derived peptides with known CD8+ T cell response profiles (2). Cells were loaded with an exogenous viral peptide mixture for 1hr before being washed extensively. After mild cell lysis, the resultant lysate was aliquoted equally and then subjected to a thermal gradient from 37℃ to 73℃. Subsequently, immunoprecipitation was carried out to capture pMHC complexes, before peptide elution and quantitation by data-independent mass spectrometry on a SCIEX ZenoTOF 7600 LC-MS/MS using an in-house spectral library of viral and endogenous peptides. ZenoSWATH DIA analysis, incorporating computational mass spectrometry and bioinformatics, allowed for thermostability profiling of >3500 pMHC, including 118 vaccinia peptides. This methodology allowed the calculation of Tm values for each peptide and provides insights into the diversity of pMHC stability across many peptide ligands. Importantly, we showed a positive correlation between virus pMHC stability and T-cell immunogenicity. The inclusion of pMHC stability information is therefore an important feature to consider when choosing potential targets for T cell immunotherapy in infection and cancer.

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id #89633

Proinflammatory cytokines improve the HLA ligandome landscape of Diffuse Midline Glioma

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Diffuse midline glioma (DMG) carries the worst prognosis of all paediatric brain tumours and is one of the leading causes of cancer-related death in children. Immunotherapy can introduce significant promise for cancer therapy. Presentation of immunogenic tumour antigens on Human Leukocyte Antigen (HLA) molecules is critical for the immune surveillance against tumours. Patients diagnosed with immunologically "cold" tumours usually have low levels of expression of HLA-peptide complexes in the tumour surface, leading to an immunosuppressive tumour microenvironment and immune evasion. Proinflammatory cytokines are involved in a "hot" tumour microenvironment encouraging immune infiltrate and have shown upregulating effect of cancer antigens along with HLA molecules. In this study, we studied the effect of two main pro-inflammatory cytokine on a) the expression level of HLA class I and II b) diversifying the HLA-bound peptides present on patient-derived DIPG-cell lines. From the 17 cell lines we studied, HLA-I expression increases by two-fold by IFN-g and 1.1-fold by TNF-a. For HLA-II, we found different patterns in different cell lines. Then by using immunopeptidomics techniques, we studied the immunopeptidome of SU-DIPG17 cell lines in the presence and absence of pro-inflammatory cytokines. In total we identified 3206 HLA-I and HLA-II bound peptides. 81 peptides were derived from known cancer-specific or associated antigens such as GPNMB and NPM. We also found IFN treatment increased the abundance of peptides present by HLA-B by 1.7-fold. However, the abundance of peptides present by HLA-A and C were less altered after IFN treatment. The results suggest that IFN-γ and TNF-α induce the expression of HLA-I and HLA-II proteins and the diversity of DMG immunopeptidome. However, IFN had a more significant effect on both aspects in both FACS and immunopeptidomics assessments. This result suggests that proinflammatory cytokines treatment enhances antigen processing leading to the presentation of novel T-cell antigens.

id #89538

Differential enhancement of antigen presentation by interferons in lung epithelia

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Publish consent withheld

id #89600

The epididymis: a window for relaying stress signals to the male germline and potential offspring

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Preconception paternal health has increasingly been linked to a spectrum of offspring health outcomes. Indeed, preclinical models have demonstrated a periconception legacy of paternal trauma that manifests in offspring as impaired stress-relevant behavioural and physiological responses. Our group previously reported that a chronic low-dose corticosterone challenge of male mice produced increased anxiety-relevant behaviours in offspring. Although sperm passage through the male reproductive tract (epididymis) is proposed to be a putative staging point for relaying stress signals to the male germline, the intricacies of the molecular pathways responsible for this form of communication remain to be fully elucidated. To address this important knowledge gap, here we have capitalised on recent advances in phosphoproteomic analyses to investigate the impact of corticosterone supplementation and consequential corticosteroid receptor downstream signalling in a tractable epididymal epithelial tissue culture system (mECap18 cells). In agreement with no overt change in glucocorticoid receptor protein (NR3C1) levels, we detected only subtle adaptation of the global proteomic profile of mECap18 cells to corticosterone challenge (i.e., 73/4171 proteins). By contrast, ~10% of the mECap18 phosphoproteome was substantially altered following corticosterone exposure. In-silico analysis of the dysregulated parent proteins revealed an activation of pathways linked to DNA repair and oxidative stress responses as well as a reciprocal inhibition of those associated with organismal death. Notably, corticosterone also induced the phosphorylation of several proteins linked to the biogenesis of regulatory microRNAs. Accordingly, orthogonal validation strategies confirmed an increase in DNA damage and an altered abundance profile of a subset of microRNAs in corticosterone-treated cells. Further, we demonstrated the DNA damage burden incurred by corticosterone can be ameliorated via prior and selective kinase inhibition. Together, these data confirm that epididymal epithelial cells are reactive to corticosterone challenge and that their response is tightly coupled to the opposing action of cellular kinases and phosphatases.

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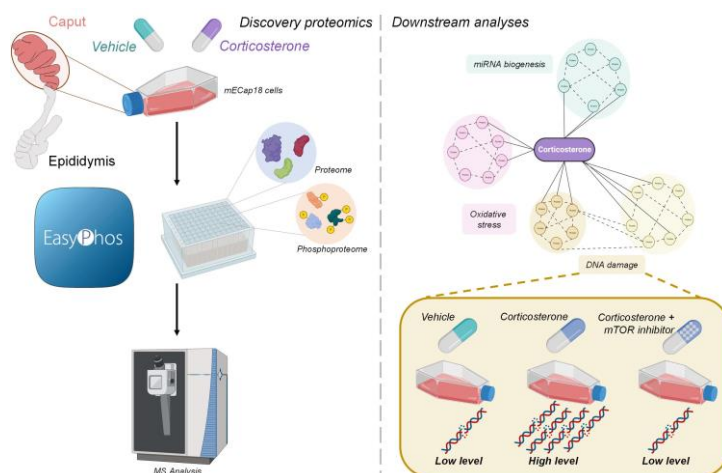
id #89372

Rapid identification of human alloantibody binding structure to human leukocyte antigen allele HLA-A*11 by cross-linking mass spectrometry.

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Antibody-mediated rejection of clinical transplantation is thought to arise from donor specific antibodies (DSAs) targeting human leukocyte antigen (HLA). Detection of anti-HLA DSAs is typically dependent on the single antigen bead (SAB) assay, where binding of alloantibodies to individual HLA alleles is measured by mean fluorescence intensity readout. The reactivity between alloantibody and HLA alleles can then be used by in silico methods to predict eplets, HLA residues which are predicted to be involved in alloantibody recognition and binding. Some recent studies have tried to identify the structural basis for alloantibody binding to HLA, including solving the crystal structure of an alloantibody to HLA-A*11:01 allele. However, the large number of possible alloantibody sequences makes it impractical to obtain high-resolution structures for every possible alloantibody to HLA allele interacting pair. Instead, ab initio-based methods and prediction-based methods, combined with rapidly obtained low-resolution structural information could provide the required information. We demonstrated a hybrid structural modelling approach using in vitro cross-linking mass spectrometry data combined with predictive methods to model the antibody-HLA interaction. Cross-linking mass spectrometry was performed using both discovery and targeted-based approaches to obtain highly reproducible cross-link data. The obtained low-resolution in vitro cross-link information was then used together with predicted antibody F_v structure, predicted antibody paratope and in silico predicted interacting surface to model the antibody-HLA interaction. This hybrid structural modelling approach closely recapitulates the key interacting residues from a previously solved crystal structure of an alloantibody-HLA-A*11:01 pair. These results suggest that a predictive-based hybrid structural

modelling approach supplemented with cross-linking mass spectrometry data can provide functionally relevant structural models to understand the structural basis of antibody-HLA mismatch in transplantation.

id #89394

Monash Proteomics & Metabolomics Facility: Offering analytical solutions to biological questions

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The complexity of the proteome is vast but remains eclipsed by the number of methods used to access its crucial biological information. Practitioners within leading labs in the world focus heavily on specialised workflows that are further refined to specific biological contexts. Core facilities aim to offer methods that are routinely applied to these researchers' needs.

Our mission at the Monash Proteomics & Metabolomics Facility is to provide the best possible proteomic (metabolomic and lipidomic) support for researchers, clinicians and the broader community by specialising in both collaborative and fee-for-service engagements. To that end, we offer standardised pipelines for the analysis of diverse sample types, from general analysis and advice to the implementation of novel approaches and applications. Our major workflows include basic identification, label-free quantification (DDA & DIA), automated bead-based enrichment, label-based quantification, crosslinking, meta-proteomics, post-translational modification analysis (including phosphorylation) and targeted methods for those crucial experiments where sensitivity and specificity are needed. Not only do we offer sample processing, MS acquisition and bioinformatic data analysis, but we also provide in-house developed tools for the study of the complex proteomic outputs from experiments, further supporting researchers in their pursuit of developing new knowledge. This is a part of our ongoing development of the *Analyst-Suite,* which includes the ability to qualitatively analyse experimental data alongside label-free quantitative analysis from MaxQuant/Fragpipe (*LFQ-Analyst*), phosphoproteomic data (*Phospho-Analyst*) with future programs of *TMT-Analyst* and *DIA-Analyst*. Here we share information on our pipelines and the depth of analysis achievable, exemplified by representative projects analysed within our facility

id #89398

A mass spectrometry quality control pipeline to enable clinical proteomics

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Introduction:

A major impediment in introducing mass spectrometry (MS)-based proteomics into a clinical setting is the lack of validation studies demonstrating reproducibility on a high-throughput scale. Standardisation through defined quality control measures and use of analytical standards are required for MS-based proteomics to be implemented in the clinic. Here we aim to develop a quality control pipeline (QCP) suitable for managing future clinical grade workflows.

Method:

We evaluated the effectiveness of quality control measures used in generating reproducible high quality MS data. Over a 4-year span >85,000 MS files (~43,000 samples, ~31,000 BSA standards and ~7,000 HEK293 MS standards) were acquired in a single laboratory across 6 instruments. The quality control measures encompassed sample preparation standards, both simple and complex MS standards, instrument-specific MS1 and MS2 thresholds, LC stability tolerances and an automated search result pipeline. All complex samples were acquired on six SCIEX 6600 Q-TOFs with 90 min runs in data independent acquisition (DIA) mode with microflow LC gradients.

Results:

The effectiveness of the QCP measures is shown by an average of 5,500 protein IDs per run obtained from ~10,000 cancer patient MS acquisitions over a 6-month period across six instruments. High reproducibility was demonstrated in a series of longitudinal replicates comprised of 46 cancer samples run in technical replicates (n=3 to 6) from 1 week up to 3 years apart. The resulting replicates maintained a per sample correlation of >0.85 and remained clustered with unsupervised hierarchical clustering.

Conclusion:

Our QC standards and QCP enables high-throughput collection of MS data to a standard that will facilitate translation of this data into a clinical setting. The data demonstrates the quality of the results achievable with the real-world implementation of such a QCP over a period of 4-years and across more than 85,000 MS acquisitions.

id #89120

Targeted proteome measurement of seed storage proteins across genetically diverse lupin varieties for identification of hypoallergenic varieties

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The demand for complementary plant-based protein sources is on the rise. This growth has been driven by population growth, consumer demand and a groundswell of interest in planetary health. The nutrient-dense grain legumes offer a sustainable solution to global food and protein security, among which the lupin seeds stand out owing to their excellent nutritional and nutraceutical properties. These seeds are one of the richest natural sources of protein and fiber and are known to be beneficial in prevention of type-2 diabetes, obesity, and cardiovascular disease. Despite their vast potential lupin seeds are under-utilized as a food ingredient and are mainly grown as green manure and stockfeed. One constraint to widespread adoption of lupin in food products owes to its allergen known content rendering it subject to mandatory labelling on food products in many countries.

Herein, a combination of discovery and quantitative proteome measurements were employed for evaluation of the four major seed storage protein families (α-, β-, δ-, and γ-conglutins) across a panel of 46 genetically diverse narrow-leafed lupin (NLL) varieties. These measurements allowed the differentiation and quantitation of the 16 known conglutin sub-families. Distinct differences were observed in the abundance of conglutins within the analyzed lines, wherein a larger variability was noted for the β- and δ-conglutin proteins. Importantly, the β-conglutin proteins, which are the major allergens from lupin, were substantially reduced within several of the domesticated cultivars, wherein some degree of compensatory elevation of the bioactive γ-conglutin proteins was noted. These potential hypoallergenic lupin genotypes are more suited to be cultivated for food purposes or prioritized in breeding programs for development of lupin varieties with optimal nutritional composition. The outcome of this research opens new vistas for further exploitation of the potential of this emerging legume as a complementary plant-based protein source.

id #89527

Acute treatment with antioxidant *N*-propionylglycine attenuates mitochondrial cysteine redox post-translational modifications and restores endogenous antioxidants in the diabetic heart, identified using quantitative mass spectrometry.

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Excessive production of reactive oxygen species (ROS) is a powerful shared mechanism in the pathogenesis of both cardiovascular disease (CVD) and Type 2 Diabetes (T2D). These disease states are common complications of metabolic syndrome as defined by hyperglycaemia, insulin insensitivity and obesity. The altered metabolic states promote a pathological increase in ROS, driving decreased cellular antioxidant capacity and promoting contractile dysfunction through protein oxidation. Clinically the cumulative effect is witnessed through increased risk of mortality following a single, acute cardiac event in T2D cohorts (Sarwar et al., 2010). Cysteine-containing proteins are at risk of oxidation, with damage limited by protecting these sites with antioxidant therapies. Using a rat model of T2D that combines low-dose streptozotocin (STZ) and high-fat diet, we performed longitudinal *in-vivo* echocardiography imaging at 0- (pre-diet), 4- (pre-STZ), and 8-week (pre-termination) time points to assess the development of cardiac dysfunction. T2D cohorts showed depressed left ventricular performance, as indicated by the reversal of the E/A ratio. Langendorff *ex vivo* perfusion was conducted on two groups of T2D hearts, the first perfused for 5 mins to clear the blood and define the degree of native protein oxidation, and the second, in the presence of an acute dose of antioxidant *N*-propionylglycine (MPG). With the addition of MPG, the *ex-vivo* contractile function was maintained in the T2D setting. Thiol-disulfide exchange and differential alkylation permitted examination of the T2D redox-proteome to identify potential sites afforded protection by MPG treatment. Redox-sensitive peptides were identified and quantified with isobaric labelling and liquid-chromatography tandem mass spectrometry (MS/MS), showing redox-sensitive sites within the contractile filament and mitochondria. Parallel metabolomics showed MPG rescued endogenous antioxidant glutathione in T2D cardiac tissue. This study demonstrated that restoring the redox imbalance by MPG improves cardiac contractile functionality in T2D conditions, identifying both essential sites of cysteine modifications and increased provision of endogenous antioxidants.

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id #89315

An ACF multicentre testing initiative: exposing the limitations of shotgun proteomics

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The Australasian Core Facilities (ACF) group has been established in 2019 to discuss challenges, opportunities and best practices in core facility management and mass spectrometric applications. In line with these goals, the ACF group has conducted during 2022 their first Multicentre Testing Initiative, which was aimed to (i) examine reproducibility across Australasian mass spectrometric core facilities and (ii) to identify optimal strategies and workflows – but also potential pitfalls and issues – in data-dependent acquisition (DDA) mass spectrometry, which is commonly referred to as shotgun proteomics.

To that end, two tryptic peptide samples of defined composition and differences were distributed to 20 participating mass spectrometric core facilities to be acquired in technical triplicates on any LC-MS/MS system(s) operated in DDA mode. Any acquisition method or workflow was admissible provided that (i) the linear separation gradient did not exceed 120 min, (ii) the samples were not further fractionated and (iii) less than 2 ug were injected per replicate. Following these guidelines, the ACF group has received a total 66 complete datasets acquired on a multitude of mass spectrometers and LC systems using a variety of parameters and settings. Upon receipt, all data were de-identified by ACF's data custodian, and forwarded to MassDynamics to be comparatively analysed and visualised using their standardised, automated pipelines.

In this poster we will comprehensively describe the findings of this first Multicentre Testing Initiative. We will furthermore use these benchmarking datasets to expose significant limitations of shotgun proteomic approaches, but also provide advice on seemingly optimal DDA strategies and workflows.

id #89687

ZenoSWATH an improved performance data-independent acquisition (DIA) on low-level proteomic samples.

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Identification and quantification of large numbers of proteins is important for the characterization of biological systems to gain insight into their composition and function. The ZenoTOF 7600 system is equipped with a Zeno trap that improves the duty cycle to more than 90% at the MS/MS level, enabling gains in sensitivity of 5 to 20x. Improved MS/MS sensitivity is important for single-cell and other applications in which samples are present at low-nanogram levels, as it enables more peptide and protein identifications. Here, we evaluated the performance of data-dependent acquisition (DDA) up to 500 ng of commercial digest loads and data-independent acquisition (DIA) approaches on a novel ZenoTOF 7600 system by testing loads ranging from 0.25 ng to 50 ng.

We used a ZenoTOF 7600 system with Zeno SWATH acquisition enabled in-line with a Waters M-Class LC system to determine protein identifications across varying commercial K562 tryptic digest loads. We determined protein identifications in either DDA or Zeno SWATH acquisition modes.

We used the Zeno SWATH acquisition capability with a 45-min gradient to test cell digest loads that were within the single-cell regime, such as 0.25, 0.5 and 1 ng loads. From these experiments, over 1000-1300, 1400 and 2300 protein groups were identified, for 0.25, 0.5 and 1 ng loads, respectively, and 45-55% of these identifications had a CV less than 20% when searched against a spectral library. At the precursor level for the same loads, there were 3000-4000, 5500 and 12000 corresponding precursors for the 0.25, 0.5 and 1 ng loads. We tested higher loads and identified 4200, 5000 and 6100 protein groups for 5, 10 and 25 ng loads, respectively, and 64-83% of these identifications satisfied the 20% CV cutoff. For a 50 ng load, more than 6300 protein groups were identified, of which 90% had less than 20% CV, and 56000 precursors were identified. When the data were searched against a FASTA library in library-free mode, the overall number of identifications and those at 20% CV cutoffs approach those achieved when processed using the spectral library approach.

A 200 ng and 500 ng load of K562 tryptic digest was tested in DDA mode on a Waters Acquity column, using a 180-min gradient. From these experiments, we were able to identify more than 4600 and 5000 protein groups for the two loads, respectively, with over 40000 and 50000 peptides for each load.

id #89680

Pathway-Analyst: one-click proteomic knowledge generation

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The application of proteomics to complex biological questions provides a wealth of essential information on disease and functional biology. The generation of quantitative datasets provides insights into drivers of disease and the functional perturbations that occur in various biological systems. Our facility has endeavored to generate tools for the processing of complex data outputs generated by various bioinformatics programs to enable researchers' one-click solutions for enabling robust pipeline analysis that generate meaningful results.

Our first program *LFQ-Analyst* offers basic insight into pathway enrichment and analysis. Here we aim to produce a new tool that aims at visualising biological information garnered from proteomic experiments. This will form a new addition to our *Analyst-Suite* named *Pathway-Analyst*, which will allow users to take lists of proteins generated in various manners, run enrichment analyses and present them in knowledge-centric figures to pull apart biological significance. We present various

figures that are generated in our new tool, whereby data is queried from a diverse range of repositories and presented in customizable figures that researchers can use in publications. Example figures include chord diagrams, UpsetR plots, Venn diagrams and annotated pathway maps.

id #89317

The genetic and dietary landscape of muscle insulin signalling and resistance

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Insulin dynamically modulates protein phosphorylation to promote glucose uptake into metabolic tissues. The failure of this process – insulin resistance – is a harbinger of diseases including type 2 diabetes. In humans, skeletal muscle is the most important site of insulin resistance. An individual's genetic background and diet are two of the strongest determinants of insulin sensitivity at this site. However, we lack a systematic understanding of how genetic variation and dietary quality modulate insulin phosphorylation networks in muscle, and how this culminates in insulin resistance. To address this knowledge gap we developed a method to interrogate mouse skeletal muscle *in vivo*, which leverages data-independent-acquisition-based phosphoproteomics to provide global snapshots of insulin signalling and measures insulin-stimulated glucose uptake from a single tissue. We applied this method to five genetically distinct inbred mouse strains fed a standard chow diet or a high-fat high-sucrose diet (HFD), and observed pronounced effects of genetic background and diet on muscle glucose uptake. Phosphoproteomics revealed marked rewiring of insulin signalling by genetics and diet. Of the 441 phosphopeptides that responded to insulin, half responded to insulin with different strength between strains and a quarter were affected by HFD-feeding. Genetic background modulated the magnitude and direction of nearly all HFD effects. Finally, association of signalling changes with glucose uptake prioritised phosphosites potentially involved in insulin resistance, including activating sites on the glycolytic enzyme Pfkfb2 and the nitric oxide synthase Nos3. These results demonstrate the fundamental role of genetics in shaping signalling networks, their responses to perturbation, and their phenotypic outputs.

id #89262

Finding the LMA needle in the wheat proteome haystack.

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Late maturity alpha-amylase (LMA) is a wheat genetic defect causing the synthesis of high isoelectric point (pI) alpha-amylase in the aleurone as a result of a temperature shock during mid-grain development or prolonged cold throughout grain development leading to an unacceptable low falling numbers (FN) at harvest or during storage. High pI alpha-amylase is normally not synthesized until after maturity in seeds when they may sprout in response to rain or germinate following sowing the next season's crop. Whilst the physiology is well understood, the biochemical mechanisms involved in grain LMA response remain unclear. We have employed high-throughput proteomics to analyse thousands of wheat flours displaying a range of LMA values. We have applied an array of statistical analyses to select LMA-responsive biomarkers and we have mined them using a suite of tools applicable to wheat proteins. To our knowledge, this is not only the first proteomics study tackling the wheat LMA issue but also the largest plant-based proteomics study published to date. Logistics, technicalities, requirements, and bottlenecks of such an ambitious large-scale high-throughput proteomics experiment along with the challenges associated with big data analyses are discussed. We observed that stored LMA-affected grains activated their primary metabolisms such as glycolysis and gluconeogenesis, TCA cycle, along with DNA- and RNA binding mechanisms, as well as protein translation. This logically transitioned to protein folding activities driven by chaperones and protein disulfide isomerase, as well as protein assembly via dimerisation and complexing. The secondary metabolism was also mobilised with the up-regulation of phytohormones, chemical and defense responses. LMA further invoked cellular structures among which ribosomes, microtubules, and chromatin. Finally, and unsurprisingly, LMA expression greatly impacted grain starch and other carbohydrates with the up-regulation of alpha-gliadins and starch metabolism, while LMW glutenin, stachyose, sucrose, UDP-galactose and UDP-glucose were down-regulated. This work demonstrates that proteomics deserves to be part of the wheat LMA molecular toolkit and should be adopted by LMA scientists and breeders in the future.

id #89096

Application of thermal proteome profiling in anthelmintic drug target identification

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Parasitic roundworms (nematodes) of humans, animals and plants have a major adverse impact on global health and agricultural production worldwide. There are significant challenges with controlling these pathogens due to widespread resistance to currently-used chemical treatments (anthelmintics) and a lack of commercial vaccines. As efforts to develop

vaccines against parasitic nematodes have been largely unsuccessful, there is an increased focus on discovering new anthelmintic entities to combat drug resistant worms. In this study, we conducted thermal proteome profiling (TPP) to explore hit pharmacology and to support the optimisation of a hit compound (UMW-868), identified in a high-throughput whole-worm, phenotypic screen. Using advanced structural prediction and docking tools, we inferred an entirely novel, parasite-specific target (HCO_011565) of this anthelmintic small molecule in the highly pathogenic, blood-feeding barber's pole worm, and in other socioeconomically important parasitic nematodes. The "hit-to-target" workflow constructed here is unique and provides the prospect of accelerating the simultaneous discovery of novel anthelmintic drugs whose targets are entirely selective, pathogen-specific and absent from the host.

id #89231

Unravelling the mechanism-of-action of an inflammatory dampening whey extract through data-independent intracellular proteome analysis

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BACKGROUND: The intestinal epithelium plays a significant role in human health by functioning as a barrier to prevent the influx of luminal antigens that cause inflammation. Dysfunction of the epithelial barrier may lead to poor digestive or systemic health conditions and is associated with multiple chronic conditions, including celiac disease and inflammatory bowel disease. In healthy individuals, barrier dysfunction can be triggered by environmental cues, such as oxidative stress caused by prolonged exposure of reactive oxygen species (ROS). These increased oxidative stress levels result in excessive amounts of pro-inflammatory mediators which exacerbate barrier dysfunction. Recently, we showed that a proprietary cationic bovine whey protein extract was able to reduce ROS production in an *in vitro* intestinal epithelial cell model (Caco-2).

AIM: To better understand the mechanisms-of-action, we investigated the effects of this whey extract on the whole intracellular proteome of Caco-2 cells during oxidative stress.

METHODS: Eighteen independent cell monolayers were randomly divided into four groups. Two groups were treated with the whey extract for 24 hours. Following removal of the treatment, one non-treated group and one treated group were challenged with 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) – a known inducer of intracellular ROS. After whole cell lysis and filter aided sample preparation (FASP) tryptic digestion, we applied ion-mobility-enhanced data-independent acquisition (DIA) label-free quantitative mass spectrometry (timsTOF pro2, Bruker Daltonics) to analyse the intracellular proteome of all individual samples. Various statistical approaches were used to study the proteome-wide responses upon AAPH-induced oxidative stress and the effect of whey extract treatment, by comparing the four groups: untreated unchallenged cells (negative control, n=4), untreated challenged cells (n=5), pre-treated unchallenged cells (n=3), and pre-treated challenged cells (n=4).

FINDINGS: From the raw datafiles we extracted approximately 150,000 features per sample, of which we identified over 84,600 peptides, that could be mapped to more than 7,500 non-redundant human proteins after combining all results. Protein pathway analysis provided more insight into the protein interactions and involved pathways during oxidative stress.

INTERPRETATION: By studying the full intracellular proteomes of an intestinal epithelial cell model with and without oxidative stress, we obtained a better understanding of the involved protein pathways and mechanism-of-action, which aids in investigating the inflammatory dampening effects of potential therapeutic compounds.

id #89611

Minimising intra- and inter-batch variation in large clinical proteomics cohort studies

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Mass spectrometry-based proteomics studies conducted on sizable population cohorts have immense power for biomarker discovery. As a result of the cohort size and extended periods of MS acquisition, however, undesirable intra- and inter-batch variation can occur which is both unavoidable and can obscure real biological signals and thus lead to false discoveries.

These large cohort studies therefore present unique challenges within the framework of existing data analysis pipelines which normally cater to moderately-sized cohorts. Critical evaluation of the most appropriate experimental design and selection of a proper normalization method are crucial to achieving reliable MS quantification results with low false discovery rate. Even with suitable controls in place, data analysis must reduce variability due to batch effects while keeping the true biological variation.

We have acquired data across a large dataset consisting of a plasma cohort (n >1000), which covered 7 batches of sample preparation and were acquired over a two-month period using diaPASEF acquisition method on a timsTOF Pro instrument. We modified an existing normalization approach2 taking the advantage of the spatial experimental design, spiked-in protein standards, and quality control pool samples (QC).

To evaluate our approach for eliminating unwanted variations, our method was compared to a standard Loess normalization used for small sample size proteomics and SERRF (systematic error removal using random forest) 3, a normalisation approach used for large scale metabolomics data. Our methodology significantly reduces unwanted variation, thereby maximising the statistical power and clinical potential of large-cohort, clinical studies.

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id #89248

An investigation into the neoantigenic landscape of microsatellite instability colorectal cancer using immunopeptidomics

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Microsatellite instability (MSI) results from genetic defects of DNA mismatch repair (MMR), leading to DNA replication errors that cannot be repaired. Around 15% of colorectal cancer (CRC) exhibit high-frequency microsatellite instability (MSI-H), the treatment for which still represents an unmet need. Neoantigens are highly specific and can form ideal targets for immunotherapy, with minimal off-target toxicities and the potential to prevent the recurrence of the disease. As the MSI-H phenotype can lead to high mutational burden and is a potential source of neoantigens, we aimed to identify mutant peptides isolated from human leucocyte antigens (HLA) of MSI-H cells using mass spectrometry.

Cell pellets from patient-derived MSI-H CRC cell lines, HCA7 and HCT116, were harvested and the cell surface HLA-A*02:01-peptide complexes were isolated. The peptide cargo was interrogated by LC-MS/MS using Bruker timsTOF PRO mass spectrometer coupled to a nanoElute UHPLC. Data was searched using Peaks X Pro against human proteome appended with a cell line specific mutation database derived from Exome sequencing.

We identified 80302 HLA-A*02:01-bound peptides that included 59 mutant peptides (neopeptides), 4 of which were shared mutant peptides between the two CRC cell lines. We noticed a high proportion of frameshift mutations compared to missense mutations within the mutated peptide sequences. These novel mutation-containing peptides have not been reported previously and form the basis of ongoing immunogenicity studies. The peptides identified and methodology applied have implications for future development of therapeutic interventions in CRC.

id #88219

Tandem mass spectrometry (LC-MS/MS) and Peptide Ion Intensity as a tool to measure protein abundance from the venom of Malaysian venomous snakes: New strategy to develop better envenomation treatment

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Snake envenomation has been identified as a neglected tropical disease responsible for a high morbidity and mortality rate, especially in Southeast Asia, including Malaysia (1). Consequently, the economic cost is beyond the capability of most low and middle-income countries. The only treatment against snake envenomation is antivenom produced from horses; however, it is associated with many drawbacks, including anaphylactic reactions and serum sickness (2). These drawbacks warranted new efforts and studies to improve the current envenomation treatment. Snake venom is a complex mixture of proteins and polypeptides with various biological activities. Information on the venom proteome is essential for understanding and predicting the clinical consequences of envenomation and formulating effective treatment/and antivenom that will neutralize toxic venom components. Advancements in proteomic techniques, i.e., shotgun proteomics and tandem mass spectrometry (LC-MS/MS), and advanced proteomic software have allowed better identification and characterization of venom proteins. Based on these techniques, we have successfully characterized venom proteomes from *Cryptelytrops purpureomaculatus* and *Tropidolaemus wagleri* (3) and have assisted in the purification of venom proteins with anti-cancer activities such as L-amino acid oxidase (4,5).

In this present study, we aimed to determine the abundance of different proteins from the venom of five venomous snake species of medical importance in Malaysia (*Naja kaouthia, Naja sumatrana, Ophiophagus hannah, Calloselasma rhodostoma*, and *Cryptelytrops purpureomaculatus*) using LC-MS/MS and peptide ion intensity approach. Although the enzymatic activities and the proteomic characterization of the venom from all these species are available, none has determined the protein

abundance using the present approach (3, 6-8). With PEAKS (PEAKS Studio, Bioinformatics Solution Inc., Canada), the relative protein abundance was measured using the peptide ion intensity feature available in the software. Phospholipase A_2 was identified as the common venom protein family in all five species, with the highest percentage identified in *C. purpureomaculatus* venom (19.78%). L-amino acid oxidase, snake venom metalloproteinase, snake venom serine protease and peptidase were identified as common venom protein families in all species except *N. sumatrana,* with varied relative abundance. The outcome of this study provides new data and new strategies can be formulated to develop an effective treatment against snake envenomation based on venom protein abundance from different species. New drugs can be created, and available drugs that target specific venom proteins found to be abundant in this project can be used as an alternative to the traditional antivenom.

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id #88895

Enhanced proteoform characterisation using an integrated top-down and bottom-up mass spectrometry approach

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The individual molecular forms of proteins – *proteoforms* – vastly outnumber genes. Since every proteoform holds potential to function in a unique capacity within the cell, effective proteoform characterisation methodologies will play a crucial role in driving systems-level understandings of cellular processes.

Despite this, the dominant analytical paradigm in proteomics, the *bottom-up* strategy, is generally ineffective when it comes to characterising individual proteoforms. This can be because the proteolytic peptides that are characterised in the bottom-up strategy are often shared across proteoforms, and/or individual modifications to the amino acid sequences are not measured. To circumvent this issue, *top-down* strategies that analyse entire proteoforms have been shown to be effective.[1] However, these strategies generally require prior knowledge of the post-translational modifications, sequence variations, and other sources of molecular heterogeneity from which the proteoforms under analysis are derived, which can limit their utility.

In the present work, we describe a proteoform characterisation workflow that integrates the individual strengths of the top-down and bottom-up approaches, while overcoming their abovementioned limitations. Firstly, the workflow leverages the capacity for bottom-up proteomics to provide non-hypothesis driven discovery and localisation localise amino acid modifications. By subjecting samples containing multiple proteoforms to bottom-up analysis using Mascot error-tolerant sequence database searches, evidence for any chemical or post-translational modifications, amino acid substitutions or other protein modifications listed in the Unimod database are uncovered and localised to specific amino acids.[2] Following this, the workflow employs top-down proteomics to measure the intact and fragment ion masses of the proteoforms under analysis. The fragment ions from the resulting data are assigned to both terminal and internal fragments using ClipsMS,[3] with the moieties uncovered in the bottom-up analysis specified as variable modifications on the specific amino acids to which they were localised. The resulting information, together with the intact mass data, enables the co-existence of specific moieties on individual proteoforms, and thus each individual proteoform's identity, to be thoroughly characterised.

We demonstrate this workflow using a sample of human full-length 2N4R tau (MAPT) proteoforms, subjected to bottom-up analysis on a Q-Exactive HF-X mass spectrometer and top-down EThcD analysis on a Tribrid Fusion Lumos mass spectrometer. Tau proteoforms derived from various combinations of cysteine to serine mutations are thoroughly characterised from the resulting data, providing proof-of-concept that our workflow can enable comprehensive proteoform characterisations without prior knowledge of the amino acid modifications or other alterations by which the proteoforms are defined.

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id #89354

Phospho-Analyst visualising and analysing phosphoproteomics data with one click

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Mass spectrometry (MS) is one of the most widely used techniques to characterise proteins and their phosphorylation status in biological and clinical samples. In a typical proteomic workflow, isolated proteins are digested into shorter fragments, known as peptides, which are analysed in a mass spectrometer. A phosphoproteomic workflow is very similar to a conventional bottom-up proteomic workflow with the exception that phosphorylated peptides are typically enriched prior to mass spectrometric acquisition using IMAC (immobilized metal affinity chromatography) or MOAC (metal oxide affinity chromatography). Software packages are then used to identify the (phospho)peptide sequences, quantify their abundance, and specific algorithms merge the peptide information to report protein-level information. Despite the increased utilization of (phospho)proteomics in biomedical research, there is still a lack of simple and user-friendly tools to support downstream analysis and interpretation. This poster presents an automated pipeline to visualise and interrogate phosphoproteomics data in the form of a web-platform called "Phospho-Analyst". It is an easy to use, interactive web application developed to perform differential expression analysis on both peptide and protein-level data. Phospho-Analyst offers a wealth of analytic features to facilitate statistical, exploratory and comparative analysis of different levels of proteomics datasets. Users can also download results in the form of tables and graphs along with auto-generated reports of key experimental outcomes. Phospho-Analyst can be accessed at https://phosphoanalyst.erc.monash.edu/.