1

Chemical proteomics reveals the target landscape of clinical kinase inhibitors

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Protein kinase inhibitors have become important cancer therapeutics, more than 30 have been approved for use in humans and more than 250 molecules are undergoing clinical trials. Polypharmacology is commonly observed for these drugs requiring thorough target deconvolution to understand their mechanism of action. Here, we report on the target spectrum of 242 clinical kinase inhibitors and 320 protein kinases using a quantitative and dose resolved chemical proteomics approach in cancer cell lines. The data revealed many novel targets for existing drugs, provides a view on the currently druggable kinome and further off-targets and suggests novel potential applications in immune or cancer therapy. Integration with phosphoproteomics data can be used to define the core pathways affected by kinase inhibitors providing a rational for combination treatments. We show that Cabozantinib may be re-purposed to treat FLT3-ITD positive AML patients and identify MELK as a novel survival marker and potential actionable target in NSCLC. All data is freely available in proteomicsDB to aid in basic and translational clinical research and kinase drug discovery.

2

CIS is a potent checkpoint in NK cell-mediated tumor immunity

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The detection of aberrant cells by natural killer (NK) cells is controlled by the integration of signals from activating and inhibitory ligands and from cytokines such as IL-15. Here we have identified CIS (encoded by Cish) as the critical negative regulator of IL-15 signaling in NK cells. Cish was rapidly induced in response to IL-15 and deletion of Cish rendered NK cells hypersensitive to IL-15, as evidenced by enhanced proliferation, survival, IFN-γ production and cytotoxicity towards tumors. This was associated with increased JAK-STAT signaling in NK cells in which Cish was deleted. Correspondingly, CIS interacted with the tyrosine kinase JAK1, inhibiting its enzymatic activity and targeting JAK for proteasomal degradation. A mass spectrometry–based approach quantifying changes in active JAK levels was used to confirm the elevated JAK1 activity and to examine the selectivity of the CIS-deficient effects. Cish−/− mice were resistant to melanoma, prostate and breast cancer metastasis in vivo, and this was intrinsic to NK cell activity. This study has uncovered a potent intracellular checkpoint in NK cell-mediated tumor immunity and holds promise for novel cancer immunotherapies directed at blocking CIS function.

Efforts are now underway to map the precise interactions between CIS and JAK1 in order to better understand how the complex can be targeted by small molecules to improve the NK cells' response to the growth factor and help patients fight cancer with their own immune system. We are using cross linking mass spectrometry to define the CIS/JAK1 binding interface.

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3

Mass spectrometry-based characterisation of protein methyltransferase substrate recognition motifs

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Lysine methyltransferases are known to regulate diverse cellular processes. Through post-translationally methylating proteins, they can modulate protein-protein interactions. While numerous lysine methyltransferases have been discovered, the mechanisms by which they select their substrates remain largely uncharacterised. Some lysine methyltransferases recognise lysines within a specific linear sequence motif, however current techniques for characterisation of these motifs are laborious and time consuming. Here we have developed a mass spectrometry-based technique to elucidate methyltransferase substrate recognition motifs, which we have applied to yeast elongation factor methyltransferase 1 (Efm1). Firstly, peptides containing alanine substitutions around the target lysine are methylated by the methyltransferase *in vitro* and analysed by LC-MS/MS. The relative methylation of each peptide is quantified in order to determine important residues in the motif. Secondly, peptide mixtures

representing all 20 amino acid substitutions at one important position in the motif are methylated *in vitro* by the methyltransferase and analysed by LC-MS/MS. Since methylation has the same mass shift as many different amino acid substitutions, a deuterated form of the methyl donor is used for this methylation reaction, conferring a unique mass shift of 17 Da to methylated peptides. The relative amount of methylation of all 20 amino acid substitutions can then be analysed in order to determine which amino acids are preferred or tolerated by the methyltransferase at a certain position within the motif. Lastly, this motif can then be searched across the proteome in order to identify potential new substrates of the methyltransferase. Application of this technique to yeast Efm1 revealed a core Y-K-X-G-G-I motif. Efm1 is only the second non-histone lysine methyltransferase to have its recognition motif elucidated, and its motif is uniquely hydrophobic among the described lysine methyltransferase motifs. This demonstrates the usefulness of this technique for the elucidation of substrate recognition motifs of methyltransferases and other protein-modifying enzymes.

4

Function of Reelin and GM1 gangliosidase during neuronal differentiation of stem cells

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The incidence of neurological disease in the population is of growing concern, however the mechanisms underlying the etiology of a host of neurological diseases remain ambiguous. Current evidence implicates disturbances of signalling pathways during neurodevelopment as a causative factor, however a robust model for studying neurodevelopment is required. This study aimed to utilize the neuronal differentiation of mesenchymal stem cells as a model for neurodevelopment, in order to analyse the expression and function of the proteins Reelin and GM1 gangliosidase, the malfunction of which are correlated with an increased risk of schizophrenia, Alzheimer's Disease, and Parkinson's Disease. Cell samples and secretions were collected at various time points during neuronal differentiation of adipose-derived stem cells, and underwent proteomic analysis via shotgun LC-MS/MS, BN-PAGE, Western blotting, and Bioplex multiplex immunoassay. Reelin and proteins pertinent to Reelin signalling and neuronal migration were detected, whilst GM1 gangliosidase and proteins relating to ganglioside catabolism were also observed. The upregulation of neuroprotective cytokines and limited expression of pro-inflammatory cytokines is consistent with literature indicating their role in signalling pathways during neurodevelopment. Together, this data shows potential Reelin and GM1 gangliosidase signalling during neurodevelopment, and validates neuronal differentiation of adipose-derived stem cell as a neurodevelopmental model.

5

Phosphoproteomic screen of exercise mimetics reveals extensive drug interactions and signalling networks

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Exercise improves health through adaptive metabolic and mechanical remodelling, mediated by a network of kinases in response to homeostatic stress. A comprehensive understanding of these signalling pathways will translate into improved strategies for the development of exercise mimetics to treat a range of diseases. Consequently, an *in vitro* model of exercise is required to delineate these signalling networks, kinase-substrate relationships and interrogate functional phosphorylation. We utilised a novel high-throughput phosphoproteomic screen to measure the action and interaction of candidate exercise-like treatments in rat L6 myotubes. Exercise-like treatments were selected if they activate a known or potential exercise kinase, or provide a stress similar to exercise. A total of 21 exercise-like treatments were initially screened and subsequent phosphoproteomic analysis using single-shot LFQ was performed on a total of 67 individual samples (10 treatments including paired controls, n=3-5). This quantified 20,249 unique Class I phosphopeptides, of which 37.8% were regulated in at least one treatment. Statistical and comparative analysis revealed the treatments regulate diverse modules in the phosphoproteome. Kinase-level analysis demonstrated drug action and provided a resource for future *in vitro* kinase-substrate and functional studies. Cross-species mapping revealed that 74% of the regulated phosphosites in exercised human skeletal muscle that mapped to the rat experiment were regulated in at least one treatment. Through enrichment analysis and a novel scoring system, it is evident that isoproterenol, thapsigargin and the combination of isoproterenol and berberine most closely recapitulate the exercise phosphoproteome. The *in vitro* combination of isoproterenol and berberine demonstrated the extent of interactions between candidate exercise-like treatments, as >200 phosphopeptides were uniquely regulated by the combination. This reveals the importance of considering drug interactions, given the range of stimuli involved in physiological processes, such as exercise.

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6

I*n vivo* application of a novel proteomic method for identifying protein targets of oxidative stress: Protein thiol oxidation in the mdx mouse model of Duchenne Muscular Dystrophy

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Oxidative stress, caused by reactive oxygen and nitrogen species (RONS), is widely recognized as important in both in the pathogenesis and subsequent pathophysiological sequelae of many diseases, including diabetes, cancer, and muscular dystrophy. We have developed a technique using novel molecular probes in conjunction with mass spectrometry to identify targets of oxidative stress *in vivo.* This proteomics-based approach allows a greater examination of the effects of oxidative stress on many proteins, facilitating insights into the mechanisms through which RONS cause functional changes to muscle tissue in Duchenne Muscular Dystrophy (DMD).

A key target of RONS is the thiol moiety of cysteine residues in proteins. This reversible oxidation has been established as a modulator of protein function. A characteristic of DMD is the loss of muscle function. We have identified significantly increased protein thiol oxidation on several proteins in the mdx mouse model of DMD. Of particular interest is the observed oxidation of the large protein titin, the oxidation of which is known to modulate the passive stiffness of muscle. As muscle stiffness affects contractile strength, this has the potential to increase susceptibility to exercise-induced damage. Titin has not been extensively studied as the large size of the protein renders it inappropriate for traditional gel electrophoresis. This finding exemplifies the value of proteomic, *in vivo* approaches to examining the effects of oxidative stress in disease models.

7

Understanding anti-inflammatory properties of dried sugarcane extracts by proteomics

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Chronic inflammation involves dysregulation in the synthesis of pro-inflammatory mediators which are associated with several diseases. Some natural plant products are known to possess anti-inflammatory properties based on their high content of antioxidants. In this study, we demonstrate that ethanol extract of whole dried sugarcane (WDS EE) is a potent source of polyphenols, flavonoids and antioxidants with high free-radical scavenging activity. *In vitro* studies performed in a cellular model of intestinal inflammation using LPS-stimulated SW480 colon cancer cells show that WDS EE supresses the phosphorylation of the NFκB transcription factor, concomitantly reducing protein expression and secretion of the IL-8 chemokine.

Mass spectrometry based phosphoproteomics enabled comparison of WDS EE with the well-known plant polyphenol, resveratrol (RSV), which demonstrated both overlapping and independent cellular responses. For WDS EE we observed the deactivation of inflammatory-modulators PKA, PKC, EGFR and c-Jun. Kinase enrichment analysis and subsequent inhibitory studies confirmed a key role of CRAF kinase in mediating WDS EE activity. Further studies using SWATH-MS quantitated 2944 proteins across all samples and subsequent bioinformatics predicted that WDS EE down-regulates NFκB pathway members including TLR2, TLR4, NIK, IκBα, and iNOS, while RSV has its key activity through members of the PI3K pathway. Overall, our findings show that WDS is a source of polyphenols that acts differently to RSV and reduces some mediators of inflammation in epithelial cells.

8

Changing medicine: Proteome centric precision health

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Precision health requires success in two intertwined aspects: precision therapy and personalized medicine. Precision therapy is being able to effectively treat the right disease; to have therapies that target for the correct pathological pathways. Personalized medicine requires diagnosing a specific individual's disease based on accurate assessment their complex health and pathological status. Our underlying premise is that an individual's baseline proteome reflects their past and present and thus, will dictate their future health and disease. We have combined tracking citrullinated proteins, an irreversible post-translational modification that can drive disease dysfunction as well as induce autoantibodies. The ability to quantify citrullinated peptides by mass spectrometry has been improved by combing searches against a library comprised of the maximal number of citrullinated peptides, data independent acquisition and downstream analytes. We have been able to identification and precise quantification of proteins and their modified forms in 5 targets organs in 4 different diseases. As well, we are determining which are also acting as autoantigens which could compound their role in disease over time. This work has led us to consider the need for continuous patent-centric health screening. We have developed technical pipelines for patient screening. This will/has required development of microsampling device, point of service devices, pathways for client data return and specific clinical grade assays. We have begun down this path with production of system suitability and quality control measures, assays and volumetric sampling device and will discuss the remaining challenges involved and requirement.

9

Digestion-free targeted LC-MS quantification of circulating Thymosin beta 4 in heart failure

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Tryptic Digestion is a necessity for big proteins, to overcome signal dilution due to multiple charge envelope and wide isotopic distribution when doing quantification by MRM. For smaller proteins, avoidance of digestion step could preserve peptidoforms information, and minimise variability. Thymosin beta-4 (TB4) is an X-linked gene product with cardioprotective properties. TB4 is a good candidate for developing a digestion-free LC-MS quantification, as it is not excessively big (approximately 5 kDa), very soluble, and is relatively abundant. We developed a digestion-free dilute-and-shoot method to quantify TB4 in plasma, then piloted the assay in heart failure (HF) cohort.

MRM for TB4 was developed empirically from synthetic standards, standard curve prepared by diluting in rabbit plasma. Plasma samples were spiked with heavy isotope internal standard, then “crashed” with acetonitrile. The assay was piloted in a nationwide heart failure cohort (n=438) with controls (n=219).

In HF patients compared to controls, plasma TB4 was significantly elevated [1265 (638–2146) ng/mL vs. 985 (421–1723) ng/mL, p=0.002]. Elevation seems to be primarily driven by women with heart failure with preserved ejection fraction (HFpEF) [1623(1040-2625) ng/ml]. Over the two years follow-up period, there were 60 deaths among patients with HF. Adjusted for NYHA class, N-terminal pro-B-type natriuretic peptide, age, and myocardial infarction, hazard ratio to all-cause mortality is significantly higher in women with elevated TB4 (1.668, p=0.036), but not in men (0.791, p=0.456) with HF. By also doing pairwise correlation of the TB4 measurements with biomarker information obtained from a multiplex proximity extension assay. We found that TB4 is strongly correlated ($R > 0.7$, $p<<0.001$) with a cluster of seven markers, six of which are either X-linked or regulated by sex-hormones. Given that the limited therapeutic options and poorly understood pathophysiology of HFpEF, our findings could lead to novel hypothesis.

10

Quantitative proteomics reveals novel diagnostic markers of Parkinson’s disease

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The combination of new bioinformatics approaches with high throughput proteomics are increasingly important across many areas of biological research. In particular, discovering new biomarkers can help in the unveiling of molecular mechanism of disease and additionally has enormous potential to enhance clinical applications in early detection and prognostic classification. Although biomarkers discovery has been a central focus of proteomics over the past few decades, many discoveries fail to validate in independent patient cohorts and as a result are not useful in clinical applications. For these reasons discovery of reliable molecular markers of disease is still a daunting task. In this work we propose that in most diseases biomarker discovery is not likely to deliver a single protein marker capable of predicting, prognosticating, or tracking disease progression. Instead, biomarkers if they exist, are likely to be complex and dynamic. Here we suggest that proteins in circulating blood exosomes contain fingerprint proteoforms that can reflect the state of the releasing cells, such as degenerative neurons undergoing Parkinson's disease. We use the Random-forest machine learning algorithm to identify a group of peptides that are capable to efficiently classify patients versus control samples. Once identified, these biomarker peptides will form the core of a ‘tool box’ for ongoing and continuous classification of Parkinson’s disease blood samples for patient diagnosis, stratification, prognosis and therapy response.

In this work we used to different batches of purified exosomes: a first from a group of 62 Parkinson’s disease patients and case controls (spouses), while a second set of samples was a larger cohort of 132 samples. Analysis of these two cohorts have showed consistent signature and preliminary analysis of the identified peptides suggest that many of them belong to proteins involved in cytoskeleton integrity and endoplasmic reticulum stress.

11

An immunoproteomics approach for the identification of novel influenza B T cell epitopes.

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Although influenza B viruses (IBVs) can contribute over 50% of seasonal influenza infections and cause severe respiratory disease, investigations of T cell responses to influenza have largely focussed on influenza A viruses (IAVs). To date only a handful of IBV derived T cell epitopes have been identified, primarily through epitope prediction or homology to IAV. Here we used mass spectrometry for unbiased identification of IBV peptides presented by HLA class I and II molecules for functional investigation. We infected human B-lymphoblastoid cell lines with IBV and sequentially immunoaffinity purified HLA-peptide complexes to yield peptides bound to (1) the common Caucasian class I allotype HLA-A*02:01, (2) the remaining class I allotypes HLA-C*04:01 and B*35:03, and (3) the class II HLA molecules (HLA-DRB1*12:01, DRB3*02:02, DQB1*03:01 and DPB1*04:01) of these cells. Eluted peptide ligands were analysed by LC-MS/MS. We identified 71 HLA class I ligands of 8-13 residues length which spanned all proteins in the IBV proteome, except the ion channel BM2 (10/11 proteins). Of these, 58 were assigned as HLA-A*02:01 ligands, commonly displaying hydrophobic HLA-A*02:01 anchor residues at P2 and the C-terminus, and 4 as HLA-C*04:01 ligands, predominantly bearing aromatic or aliphatic residues at P2 and the C-terminus, and Asp at P3. A further 212 HLA class II ligands, principally 12-18 residues in length, were identified from 6/11 IBV proteins. Notably, although dominated by hemagglutinin, HLA class II ligands were also sourced from BM2, suggesting compartmentalisation to the HLA class II loading pathway. Preliminary functional analyses in HLA-A*02:01$^+$ individuals and HLA-A*02:01 transgenic mice determined the immunogenicity of a number of the HLA-A*02:01 IBV derived peptide ligands identified, confirming the potential of this approach to provide candidate peptides for investigation of IBV-specific T cell responses.

12

What Proteomics can tell us about Platelets!

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More than 130 years ago, it was recognized that platelets are key mediators of hemostasis. Nowadays, it is established that platelets participate in additional physiological processes and contribute to the genesis and progression of cardiovascular diseases. Recent data indicate that the platelet proteome comprises >5000 proteins. By implication, in patients experiencing platelet disorders, platelet (dys)function is almost completely attributable to alterations in protein expression and dynamic differences in post-translational modifications. Consequently, platelet proteomics will represent an invaluable tool for characterizing the fundamental processes that affect platelet homeostasis and thus determine the roles of platelets in health and disease.

In the past few years, it has become increasingly clear that platelet proteomics can provide novel insights into basic research questions and thus improve our understanding about the fundamental processes that regulate platelets and can also contribute to the diagnosis of platelet disorders. The protein composition and the phosphorylation patterns of platelets will be useful to understand certain disease states and therapeutic interventions. In particular, quantitative phosphoproteomic studies will pave the way for a refined understanding of platelet properties.

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A combination of omics strategies to elucidate the proteolytic networks in macrophage differentiation

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Publish consent withheld

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Lysine acetylation throughout axenic culture in the early-branching eukaryotic parasite, *Giardia duodenalis*

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Acetylation of lysine (KAc) is a ubiquitous post-translational modification, however the extent and functional impact of this modification, as well as specific acetyl-lysine sites, are largely unknown in the parasite *Giardia duodenalis*. *G. duodenalis* is responsible for over 200 million cases of diarrhoeal gastroenteritis (giardiasis) annually, and is considered an early-branching eukaryote, possessing basic eukaryotic cellular traits and systems. Here, we report the first analysis of the 'acetylome' in this parasite, by comparing acetylation data for the infective trophozoites life-stage during log, stationary and declining phases of axenic culture. Preliminary analyses identified 1747 acetylation sites among 705 unique proteins, including 487, 235 and 153 sites identified uniquely in log, stationary and declining phases, respectively. These data suggest KAc is a widespread modification in *G. duodenalis*, as acetylated proteins detected thus far constitute 9.47% of non-deprecated gene products in this parasite. Global analysis of sequence motifs indicated enrichment of lysine and neutral amino acids (glycine, leucine and tyrosine)

at positions neighbouring KAc. Functional annotation indicated that the majority of the ribosomal machinery is acetylated, as well as the TCP-1 (T-complex protein) chaperone family. A total of 80 acetylated proteins were *G. duodenalis* kinases, comprising members of *Giardia's* reduced core kinome, and the uniquely-expanded Nek kinase family. Many annexin-like proteins from *Giardia* gene families were also acetylated. These proteins are considered important constituents or accessories of the cytoskeleton, including alpha-giardins, axoneme-associated proteins, NEK kinases and members of the protein 21.1 family. Furthermore, all three peroxidredoxins, and four of five genomically encoded protein disulphide isomerases were acetylated, implicating acetyllysine in the regulation of crucial redox processes in *G. duodenalis*. This preliminary work will be expanded with additional biological replicates to quantify the reproducibly of identified KAc sites, and complimented with quantitation of the total proteome throughout axenic culture.

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Signalling networks in the regulation of red blood cell integrity and survival.

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Red blood cells (RBC) are essential for transport and exchange of O_2/CO_2 around the body. Two major multi-protein complexes are critical for RBC integrity as they bridge the lipid bilayer and connect integral membrane proteins to the cytoskeleton (composed of α/β-spectrin and actin), designated as the ankyrin complex and the 4.1R complex. Both complexes encompass the anion exchanger Band3, which is essential for CO_2 removal by exchanging chloride for bicarbonate. Its exchange activity is mediated by its C-terminal transmembrane domain, while its N-terminal cytosolic region anchors many proteins including ankyrin (linking directly to α/β-spectrin), glycophorin C, adducin, Band4.1, deoxy-haemoglobin (deoxy-Hb) and Duffy. Lyn and Syk tyrosine kinases are responsible for phosphorylating Band3 in response to stress stimulation (oxidation, osmotic and physical). However, apart from this aspect of RBC biochemistry, not much is known of signalling networks in RBCs. We have recently shown that excessive Lyn activity (*Lyn$^{up/up}$* mice) causes formation of RBC acanthocytes (RBC with thorn-like protrusions). Acanthocytes arise in several conditions when there are alterations to membrane structural proteins. Indeed, patients with chorea acanthocytosis (ChAc) display circulating acanthocytes and possess abnormally high Lyn activity in their RBCs, which is the cause of their acanthocytic morphology. Proteomic analysis of ChAc RBCs showed altered phosphorylation of Band3, ankyrin and β-spectrin, which affects cross-talk with adducin. Significantly, altering Lyn levels and activity has important effects on RBC biology in response to stress stimulation including *in vivo* half-life, *in vitro* viability and integrity, and membrane protein association. We have used several proteomic approaches to investigate the intricacies of signaling networks controlling the RBC membrane/cytoskeletal. Using quantitative iTRAQ as well as phospho-tyrosine-peptide mass-spectrometry we have identified specific pY sites in Band3, Band4.1, α/β-spectrin, and ankyrin mediated by Lyn/Src kinases as well as quantitative changes in membrane/cytoskeletal protein associations regulated by Lyn.

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A proteogenomic approach to understanding adaptive immunity in bats

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Bats are a major reservoir of emerging infectious diseases that pose a serious threat to animal and human health. Bats harbour deadly viruses such as Ebola, SARS and Hendra virus (HeV) without clinical signs of disease. Vaccines are not available against many of these viruses and a better understanding of the bat immune system could aid vaccine development. We hypothesise that the bat adaptive immune system contributes to the ability of bats to co-exist asymptomatically with viruses. One of the key adaptive immune responses is the presentation of antigens by major histocompatibility complex (MHC) class I molecules to $CD8^+$ T cells. We sought to understand antigen processing and presentation in bats by characterising the first bat MHC class I molecules using a proteogenomics approach.

We immunoaffinity-purified bat MHC class I molecules and bound proteins and peptide cargo was analysed by LC-MS/MS. We show for the first time that bat MHC class I molecules associate with a peptide-loading complex. Furthermore, we characterised the first endogenous peptide repertoires of three distinct bat MHC class I molecules. These peptides ranged from 8 to 15 amino acid residues and motif analysis revealed strong amino acid biases at various anchor positions. Lastly, we identified HeV-derived peptides from infected bat cells which also displayed similar amino acid biases. We exploited these motifs to predict viral epitopes *in silico* and 20 novel HeV peptide ligands were confirmed by LC-MRM analysis.

In conclusion, this study describes the functional characterisation of the first bat MHC class I molecules and their respective peptide repertoires. We have also identified the first viral peptides presented by bat MHC class I molecules. Our results provide fundamental insights into the antigen processing and presentation pathways of bats, which ultimately can be used to understand viral control.

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A proteomic investigation of diseases of Sydney rock oysters: A step towards breeding better oysters

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Sydney Rock oysters (SRO) reflect the Australian way of life. Their limited distribution along the coast of NSW makes them precious from both an ecological and economic point of view. In recent years, especially since the 1990s, a few diseases such as winter mortality (WM) and Queensland Unknown (QX) have impacted SRO farming greatly. Selective breeding has provided a temporary relief by reducing the mortality rate of the oysters. The selection, however, is not backed by the knowledge of genes helping the cause. In this attempt to unravel the phenomenon underlying the mechanisms of selection we have performed a proteomic analysis of WM and QX disease resistant selected oysters.

We approached the WM study using traditional 2DE in conjunction with LC-MS/MS. The results showed cytoskeletal breakdown as a result of disease stress, and we also identified novel proteins such as proteasome subunit alpha type-6 and calcium-dependent protein kinase 31. These proteins have not been reported previously in the context of disease resistance in oysters. Result from this study added to the knowledge of stress response pathways of SRO.

For the QX study, oysters sampled at different time points were used to generate proteome information and to get an idea about disease progression as well. The shotgun proteomics study identified at very high stringency ~150 reproducibly identified proteins. We have observed a notable increase in enzymes associated with energy consumption (ATP synthase), proteolysis (proteasome) in the case of non-selected oysters, which supports the previous observation regarding cytoskeletal breakdown pathway. A few proteins identified in this study, such as HMGB1, are known to be involved in immune response and inflammation, and represent promising candidates for further study.

The general idea from this study is to identify the underlying mechanism involved in selective breeding.

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Characterisation of the *Campylobacter jejuni* Cj0025c nutrient transporter using a multi-omics approach

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Campylobacter jejuni is the leading cause of bacterial gastroenteritis in the developed world, and is responsible for as many as 400 to 500 million cases a year worldwide. Human infection is predominantly caused by the consumption of improperly prepared poultry products. *C. jejuni* exists as an asymptomatic commensal organism within the intestines of chickens, but is virulent to humans. While the exact mechanisms underpinning this difference in pathogenicity remain unknown, factors such as motility and nutrient uptake are thought to play a role. Proteomics analysis of *C. jejuni* NCTC 11168 O under conditions that mimic human host environments (supplementation with deoxycholate [bile salts], the iron chelator deferroxamine, and high salt) identified Cj0025c as the most induced protein under such conditions (2.5-8.0-fold induction). Cj0025c encodes a putative sodium:dicarboxylate nutrient transporter. In order to help determine the function of Cj0025, a knockout mutant was analysed using a variety of techniques, including phenotypic and virulence assays and label-free SWATH proteomics. Deletion of *cj0025c* resulted in significantly reduced motility and invasion of human Caco-2 intestinal epithelial cells compared with wild-type. Assays employing dicarboxylates as sole carbon sources in minimal medium, combined with metabolomics, were used to identify Cj0025c-associated nutrients. Proteomics highlighted differences in protein abundance between knockout and wild-type *C. jejuni*, including those with functions in extracellular polysaccharide and lipopolysaccharide biosynthesis, possibly suggesting a role in cell-cell interactions and biofilm formation.

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Biomarker Signatures in Melanoma

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The UPR (unfolded protein response) has been identified as a key factor in the progression and metastasis of cancers, notably melanoma. Several mediators of the UPR are upregulated in cancers, e.g., high levels of GRP78 (glucose-regulator protein 78 kDa) correlate with progression and poor outcome in melanoma patients indicating the UPR promotes tumourigenesis and metastasis. AMPylation, the covalent addition of adenosine monophosphate (AMP), of GRP78 has recently been identified to modulate the UPR cascade. Utilising immunoaffinity and mass spectrometry we have identified differential AMPylation of several key UPR proteins in response to increased UPR in melanoma. The complete role of the UPR has yet to be defined. Understanding how the UPR allows for adaption to stress and thereby assists in cancer progression is important in defining an archetype of melanoma pathology. In addition, targeting AMPylation of key UPR proteins could prove an effective treatment against melanoma.

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Quantitative proteomic analysis of the exercise-induced extracellular vesicle proteome

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Proteomic analysis of plasma offers tremendous insight into tissue to tissue communication and signalling in complex scenarios such as exercise. Inspired by the growing appreciation that such cross talk might be partially mediated by proteins packaged in extracellular vesicles, we have carried out a deep analysis of the exercise-induced extracellular vesicle proteome. Using a series of centrifugation and PBS wash steps, we isolated extracellular vesicles from the arterial plasma of 11 human participants carrying out a 1hr bout of cycling. All samples were analysed by nano-ultra high-pressure liquid chromatography coupled to tandem MS on a Q-Exactive MS operated in DDA. In addition to experimental samples taken at rest, immediately and 4 hours after exercise, we also pooled exercise and baseline samples and fractionated via neutral pH HPLC on an in-house micro packed BEH C18 column to derive a deep reference proteome. Raw data were searched against the human uniprot database in Maxquant, utilising the match between runs function to map and transfer identifications from the reference data set to the experimental samples. We identified a total of 5359 proteins at an FDR of 0.01, with the large majority of this coverage derived from the fractionated reference proteome. Matching between runs resulted in a 105% gain of identifications in experimental samples, facilitating a quantitative LFQ comparison of 1159 proteins. Analysis in Perseus revealed 325 proteins to be differentially regulated by exercise with a notable upregulation of several classes of proteins that compose the canonical 40-150nm exosome. Proteins associated with the formation (ALIX), trafficking (RAB GTPases, Annexins) and target adhesion (Integrins, tetraspanins) of exosomes were all upregulated by exercise. Pathway analysis revealed significant enrichments in several biological processes and signalling pathways. This raises the possibility that exercise mediates many of its affects via the release of key signalling molecules packaged in exosomes.

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The protein landscape of the inner ear of a native fish

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Otoliths are small, bio-inorganic structures found in the inner ear of fishes. These “earstones” first form *in embryo*, and then grow incrementally through the daily deposition of alternating proteinaceous and mineral bands. As the chemistry of an individual fish’s ambient environment varies from day-to-day, each increment will then differ, specifically in the amounts of trace-elements incorporated. Ecologists routinely utilise increment trace-element ratios to reconstruct fish environmental histories. Little is known, however, about the structure and composition of the proteinaceous portion of otoliths. Due to this, there is considerable uncertainty as to whether a given trace-element is present through environmental change, or as a necessary component of one of the proteins present. To answer this question, we first sequenced the transcriptome of a southern Australian fish, *Acanthopagrus butcheri* (Black Bream). This was then matched to proteomic data collected from the separated organic phase of otoliths and endolymph (inner ear fluid) from wild, adult Black Bream. Our work presents the first transcriptome of a sparid fish, as well as identifies novel inner ear proteins present in *A. butcheri.*

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Glycoproteomics for analysis of the regulation and evolution of protein glycosylation

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Protein glycosylation is a critical post-translational modification that regulates the functions of diverse proteins in eukaryotes. However, analysis of protein glycosylation is hampered by glycoprotein structural diversity both in site occupancy (macroheterogeneity) and glycan structure (microheterogeneity). We have developed variations of SWATH-MS (Sequential Window acquisition of All THeoretical fragment ions Mass Spectrometry) to enable straightforward yet powerful global and targeted analysis of glycoprotein structural diversity. Using Saccharomyces cerevisiae as a model organism, we measured glycan occupancy and structure on a range of glycoproteins in strains with mutations in the N-glycosylation pathway. We observed different degrees of hypoglycosylation in all mutants, including in mutants in regulatory subunits of the oligosaccharyltransferase that catalyses N-glycosylation. The stronger hypoglycosylation phenotypes occurred in strains with defects earlier within the ER lumenal mannosyltransferase steps or glucosyltransferase steps, and only mannosyltransferase mutants showed extensive global changes in glycan structure. Defects in macroheterogeneity therefore did not correlate with defects in microheterogeneity, highlighting the importance of both aspects of glycoprotein structural diversity. We further used these tools to investigate the constraints of co-evolution of a single oligosaccharyltransferase enzyme with diverse glycoprotein substrates in fungi and vertebrates. These results provide a quantitative and qualitative overview of the regulation of oligosaccharyltransferase activity in the presence of truncated glycan donor substrates, and describe outcomes of defects throughout glycan biosynthesis.

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Developing a glycoproteomics tool kit using synthetic *N*-glycopeptides

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Detailed information on glycoprotein primary structure is a prerequisite for understanding their diverse functions. Aspects such as glycosylation micro- and macroheterogeneity (1) are nowadays mostly determined using MS and tandem MS approaches (2). Conquering the final frontiers in the glycospace requires also a solid understanding on glycoprotein and glycopeptide related sample preparation and MS analysis aspects. Synthetic glycopeptides offer a unique opportunity to investigate and validate glycoproteomics analyses and develop new methods.

We have developed novel and simplified approaches to produce *N*-glycan carrying amino acids for solid phase peptide synthesis. In combination with targeted chemo-enzymatic glyco-modifications these building blocks allowed us to establish a well-defined glycopeptide library containing >100 glycopeptides plus their unglycosylated counterparts. This library has been used to systematically assess glycoproteomics aspects such as HILIC glycopeptide enrichment, collision stepping CID, effective ionisation and ion-mobility glycoproteomics.

This capacity allowed us to quantify and determine the ionization suppression effect glycopeptides are experiencing compared to their unglycosylated counterparts (3). CaptiveSpray Nanobooster™ ionisation, however, can effectively enhance their ionization, providing access to compounds hardly detected by other ESI ionisation techniques. This glycopeptide library also enabled us to optimise CID/HCD/ETD, fragmentation by improving data quality for software assisted glycopeptide assignment. These optimised conditions were also validated on biologically relevant samples such as the entire panel of human Immunoglobulins (4). The solvent effect on ZIC-HILIC glycopeptide enrichment was systematically evaluated, revealing a tremendous solvent-dependability (5). These synthetic *N*-glycopeptides were also the key to develop innovative ion-mobility based techniques providing glycan structure information on sialic acid linkage directly from glycopeptide tandem MS data (6), making a huge leap towards site specific glycan structure analyses going beyond compositional data. This toolkit provides a solid basis to find answers to the functional role of protein glycosylation in health and disease.

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Revealing glycan structural epitopes using a simplified LC-MS/MS data acquisition and analysis platform for glycomics

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LC-MS/MS analysis glycans released from proteins can provide structural information such as monosaccharide composition, sequence, branching and linkages, which is important for an understanding of their role in biology. Three difficulties in characterising and quantifying these glycans is the high dynamic range of glycan structure abundance, the structural similarity between glycan isomers and the lack of a vendor-neutral, open-access tool for quantifying glycans across a large number of LC-MS/MS datasets. Here, a streamlined data acquisition and analysis platform for glycomics was applied to reduced *N*- and *O*-glycans released from membrane-bound and secreted glycoproteins extracted from drug treated neuronal cell lines.

Our data acquisition approach uses a porous graphitised carbon column to achieve liquid chromatographic separation of glycan structural isomers, allowing characteristic CID-MS/MS of each isomer to be acquired in negative mode. The use of a linear ion-trap mass spectrometer addresses the high dynamic range in glycan structural abundance by using an optimised instrument method to effectively characterise low abundance glycans. This method has provided characterisation of over seventy *N*-glycans on proteins secreted from neuronal cell lines. Glycan structural epitopes characterised include outer-arm fucosylation and oligo-sialic acid motifs which have previously been implicated in cancer and neuronal regeneration respectively.

The absence of an open access platform for label-free glycan quantification has an impact on data analysis throughput, reproducibility and data sharing. Using Skyline, a software tool used in targeted proteomics, ion chromatograms of each observed charge state of all characterised glycans were generated and corresponding peak areas were integrated automatically. The Skyline method was compared to our conventional manual analysis approach and high consistency was observed. The high throughput of this streamlined method was illustrated by the glycomics analysis of membrane-bound and secreted glycoproteins from a time-course study of human and mouse neuronal cell lines, investigating immune responses to lipopolysaccharide treatment.

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Evaluating the relationship between *N*-Glycosylation and protein stability in *Campylobacter jejuni*

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Campylobacter jejuni is a leading cause of acute gastroenteritis in developed countries, and was the first prokaryote demonstrated to extensively modify proteins by *N*-linked glycosylation. This highly conserved post-translational modification system is crucial for pathogenicity, albeit the mechanism remains to be elucidated. With over 120 modified sites identified to date, recent findings have suggested that *N*-glycosylation may be important for protein stability in various physiological and pathogenically relevant contexts. To explore this further, we employed iTRAQ-based labelling to determine the effect of either loss of the oligosaccharyltransferase (Δ*pglB*), or biosynthesis of the glycan (Δ*pglDEF*) on whole protein abundance in a relatively recent clinical isolate, *C. jejuni* JHH1. Of the 1077 *C. jejuni* proteins quantified, only 57 were deemed to have a significant change in abundance in either of the Δ*pgl* strains relative to the wild-type isolate. A large proportion of known glycoproteins were quantified with ~17% displaying an altered abundance in the *N*-glycosylation negative strains. *N*-terminal amine isotopic labelling of substrates (*N*-TAILS) was also employed for pair wise comparisons of the *N*-degradome of wild-type JHH1 and individual *pgl* deletion strains to address the hypothesis that the *N*-linked glycan may provide protection from proteolytic degradation. We were able to identify and quantify 4122 unique *N*-termini from 766 *C. jejuni* proteins. From those derived from known *N*-linked glycoproteins, a number were found to be in close proximity to or contained the sites of *N*-linked glycosylation and in turn displayed a significant difference in their relative abundance in the Δ*pgl* mutants. Further, we coupled *N*-TAILS to intact *N*-glycopeptide analyses to identify putative *N*-terminal *N*-glycopeptides. These proteomics-based approaches were complemented with various standard phenotypic tests to establish how the loss of *N*-glycosylation extended to broader changes in *C. jejuni* physiology.

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Cell-type specific profiling of specialized trichomes from the salt-tolerant plant *Mesembryanthemum crystallinum* reveals insight into their development and role in plant salt tolerance.

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Trichomes are highly differentiated cell types found on the aerial epidermis of plants. Most commonly they manifest themselves as hairs on leaves, stems and flower buds, and serve a range of functions from protection against insects to heat resistance and moisture conservation. The salt tolerant plant *Mesembryanthemum crystallinum* (common name Ice plant) has specialized balloon-like trichomes called epidermal bladder cells (EBC) that are thought to play a role in sodium storage and salt tolerance, however, EBC are present on plants even in the absence of salt. In order to gain an integral understanding of the development and role of these cells we have taken a systems biology approach, combining data from global quantitative transcriptomics, proteomics, metabolomics, and ionomics studies with flow cytometry and fluorescence microscopy. Our results demonstrate that these cells are highly metabolically active and not just passive stores for sodium. The rapid expansion of the cells during development is due to a huge increase in ploidy, beginning in emerging leaves, to higher than 512C in adult plants, with nuclei of greater than 60 microns in diameter measured in these cells. Salt-treatment leads to a further increase in ploidy and transcripts and proteins involved in endoploidy are shown to be upregulated by salt in the EBC suggesting environmental as well as developmental cues. It has been proposed that increased ploidy helps to mitigate stress damage, and the increase in cell size and ploidy observed in *M. crystallinum* under salinity may contribute to tolerance by increasing the store size for sodium sequestration. However, as significant increases in ploidy are observed in the untreated plants it is possible that higher ploidy also facilitates higher cellular metabolic activity and rapid cell expansion in this plant.

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Protein correlation profiling and the dynamics of the *Saccharomyces cerevisiae* intracellular methylation network

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Underpinning much of the research at the NSW Systems Biology Initiative (SBI) is a drive to better understand how the dynamics of protein-protein interaction (PPI) networks are regulated. In exploring this question we have devoted particular attention to the study of arginine and lysine methylation – post-translational modifications capable of altering PPIs – in the model organism *Saccharomyces cerevisiae*. We have created a foundation for this work by producing a detailed map of methyltransferase-substrate interactions embedded within a broader network of PPIs: the yeast intracellular methylation network.

This presentation will describe how we are now studying the dynamics of this network using a technique known as size exclusion chromatography-protein correlation profiling (SEC-PCP). SEC-PCP uses mass spectrometry to detect SEC fractionation profiles for individual proteins in native complexes; similar fractionation profiles are correlated under the assumption that proteins in a common complex co-fractionate.

We have created a reference PCP dataset from 280 SEC fractions of wild-type yeast lysate (70 fractions × 4 biological replicates), identifying >200 core complexes in their native form, including complexes that contain known methyltransferase substrates. We are now in process of producing PCP data from methyltransferase knockout yeast strains to study how altered methyltransferase-

substrate interactions affect PPIs. We are also characterising absolute stoichiometries of individual methylation sites in these core complexes using a library of 34 internal standard heavy isotope-labeled synthetic peptides. This has, for example, provided insight into the methyltransferase activity on EF1α – a methyltransferase substrate that we observe in at least 3 distinct core complexes – when EF1α is associated with different PPIs.

This presentation will also reflect more broadly on our efforts to create reference datasets for systems biology model organisms. In particular it will consider how underlying values driving systems biology research relate to data quality, and spread of false positive data.

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Substrates and and kinetics of Arg-GlcNAcylation by the NleB/SseK family of bacterial type III secretion sytem (T3SS) effectors

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Bacterial glycosyltransferase toxins are secreted effectors which subvert normal cellular functions to enhance bacterial pathogenesis or survival. Recently a novel glycosyltransferase T3SS effector capable of transferring GlcNAc to Arginine residues was identified in enteropathogenic *Escherichia coli* (EPEC). NleB1 was shown to modify the death domain of FADD and TRADD and block extrinsic apoptosis signaling. To establish the true repertoire and kinetics of Arg-GlcNAcylation by NleB1, we developed an Arg-GlcNAcylation enrichment strategy. Utilizing a newly developed Arg-GlcNAc specific antibody we show endogenous targets of NleB1 can be effectively enriched at the glycopeptide level enabling both identification of glycosylation substrates and sites of modification. Using inducible stable cell lines, we found that multiple human proteins can be modified by NleB1 yet FADD appears to be the dominant and first Arg-GlcNAcylated target. Temporal profiling of Arg-GlcNAcylation showed that, during EPEC infection, alternative Arg-GlcNAcylation substrates only appeared after FADD modification or in response to overexpression of NleB1. Similarly, homologues of NleB1 such as SseK1 from *Salmonella enterica* serovar Typhimurium also appeared to target FADD at the conserved residue Arg^{117}. However, within TRADD the death domain was modified at alternative sites by SseK1 compared to NleB1. Surprisingly, within both EPEC and *S. Typhimurium*, we noted the modification of multiple bacterial proteins including NleB1 and SseK1 themselves. These findings suggest FADD is the preferred target of NleB1 during EPEC infection and highlights the power of Arg-GlcNAc pulldowns for the identification and characterization of Arg-GlcNAcylating enzyme substrates.

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Quantitative shift in MHC epitope abundance during the two major pathways of antigen presentation following virus infection: implications for the induction of antiviral immunity

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The pathways of MHC class I (MHCI) antigen processing and presentation play a crucial role during the recognition and elimination of virus-infected cells by $CD8^+$ T cells. Two major pathways of MHCI processing exist, that of direct presentation whereby endogenous proteins are cleaved into short peptides for MHC-binding, or cross-presentation whereby exogenous antigens are taken up by professional antigen presenting cells and subsequently processed. Both pathways result in MHC-bound peptides being displayed on the cell surface, but the relative contribution of each pathway to T cell immunity, especially at the level of epitope abundance, remains unclear.

In the present study we used MHCI-peptide elution and LC-MRM to monitor the absolute and relative levels of influenza A virus (IAV) peptides during *in vitro* models of direct and cross-presentation. A total of 22 virus-derived MHCI-peptides were identified and quantified, including the immunodominant epitopes $NP_{366-374}$ and $PA_{224-233}$. Direct presentation revealed a diverse range of peptide abundance, with $NP_{366-374}$ being present at more than a thousand copies per cell, whereas $PA_{224-233}$ was the lowest at just 10 copies per cell. In stark contrast, cross-presentation resulted in a much narrower focus of peptide abundance at relatively low levels, with highly diminished presentation of $NP_{366-374}$ yet sustained abundance of $PA_{224-233}$ compared to direct presentation. Given that *ex vivo* $CD8^+$ T cell responses were monitored towards all peptides, these data allow correlations between epitope presentation pathways, peptide abundance and resultant T cell immunity to be explored. These novel insights into the quantitative nature of antigen presentation hold promise for the future development of antiviral vaccine strategies.

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Ultraviolet photodissociation and ion-electron reactivity of extremely supercharged protein ions

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To characterise sequence and post-translational modifications of proteins, the molecular ion and fragment ion data that is obtained by activating an intact protein ion inside a mass spectrometer (MS) can be significantly more discerning than that obtained for peptides formed by protein digestion. The primary advantage of intact protein MS is that degradation products,

sequence variants, and different combinations of post-translational modifications can be distinguished. However, complete characterisation of intact protein ions that are larger than *ca.* 10 kDa is challenging primarily because ions in relatively low charge states do not readily fragment unlike those in higher charge states. Here, we report our discoveries that (i) proteins can be supercharged beyond the theoretical limit to protein charging in electrospray ionisation (ESI), which is based on gas-phase proton-transfer reactivity, and (ii) these ions that are formed by "extreme" supercharging fragment significantly more readily than lower charge states in ion-electron recombination experiments (electron capture dissociation, ECD; *e.g.*, greater than 85% of 259 inter-residue sites can be identified in a single MS experiment for bovine serum albumin). The first results from ultraviolet laser induced photodissociation of extremely supercharged protein ions will also be reported.

"Extreme" protein supercharging was discovered by adding butylene carbonate (and structurally related analogue molecules) to ESI solutions, which resulted in the formation of protein ions in significantly higher charge states than have been reported by use of other methods/additives. ECD data was obtained using a Thermo 7 T LTQ-FT/ICR MS (UNSW). Ultraviolet laser induced photodissociation tandem mass spectrometry measurements of intact protein ions were performed using a Orbitrap Q Exactive Plus (U Melbourne), which has been equipped to allow *m*/*z*-selected ions to be irradiated with a single pulse of 193 nm photons (6.4 eV).

"Extreme" supercharging of protein ions is useful for significantly improving the sequence characterisation of intact proteins by top-down MS.

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Biomedical applications of SONAR - a novel data independent acquisition method for quantitative and qualitative analyses

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Most Data Independent Acquisition strategies utilize a stepped modes of acquisition, where the first mass filter is set to pass a fixed m/z range, with windows typically 5-20 m/z unite. The need to use a number of different steps to cover the m/z range of interest leads to a relatively long duty cycle of acquisition, which slows the analysis time and can lead to relatively poor quantitative reproducibility. In SONAR, a resolving quadrupole is scanned repetitively over alternating low and elevated energy scans. This produces data in a similar format to Ion Mobility enabled acquisitions. The relatively high speed duty cycle can be exploited to reduce analysis time and thus sample throughput. Notably, SONAR can be used with very high throughput separation systems. An additional feature of SONAR it that both conventional database searching and spectral library matching can be used to make qualitative identifications. We have applied SONAR to address several biomedical projects – changes in the synaptosome as a function of gene knock-down, and the effects of drug treatments on protein:protein complexes.

The fast duty cycle of SONAR has been shown to provide very high quantitative reproducibility, via the analysis of project-specific Study Pool QC samples. For example, in the synaptosome study, 1,712 proteins gave an overall 5.8% CV, whereas the protein:protein interaction QC pools gave 7.49% CV. In addition to the high quantitative reproducibility, these experiments show SONAR provides information-rich datasets, with a qualitative and quantitative dataset acquired for each sample. Since SONAR does not require a DDA spectral library, the datasets can be directly searched against a FASTA database.

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MS1 based quantification optimization on DIA methods on a quadrupole-Orbitrap mass spectrometer

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Introduction

Targeted analysis of DIA is a powerful mass spectrometric approach for comprehensive, reproducible and precise proteome quantitation. It provides valuable insight into biological processes or enables the discovery of novel biomarkers. Today, identification of the majority of the expressed proteins can now be achieved. With these achievements in the identification, the reproducibility and quantitative accuracy and precision have become increasingly important. Here, we optimize data-independent acquisition (DIA) on a Thermo Scientific™ Q Exactive™ HF mass spectrometer for quantification at the MS1 level. DIA data can be processed in a targeted mode based on MS2 fragment information. MS1 scans have potentially a higher sensitivity since the peptide precursor is not fragmented in multiple fragments. Novel methods using high resolution full range and segmented MS1 enable high precision quantification on MS1. Here we perform quantification of over 60,000 peptide precursors on MS1 level and benchmark MS1 based interference correction implemented in Spectronaut.

Methods

Protein samples for HeLa were prepared using the FASP protocol. Biognosys' iRT kit was spiked into the samples before injection. The samples were acquired on a Q Exactive HF mass spectrometer. Shotgun runs were performed and searched using MaxQuant software. Spectral libraries were generated with Biognosys' Spectronaut. DIA methods were acquired with varying gradients lengths. Targeted analysis of DIA runs was performed using Spectronaut.

Novel Aspects

Single shot DIA method using high resolution MS1 scan information for quantification.

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Proteome-wide drug dose-response of prostate cancer cell lines exposed to androgen receptor antagonists by microflow-LC SWATH MS analysis

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Many proteomic studies rely on the steady-state analysis of tissues or cell lines to identify markers of diseases or predict drug treatment outcome. In many instances, however, proteome changes upon drug exposure may be better suited to clarify in-cell drug action, identify off-targets or reveal mechanisms involved in drug resistance. Complete drug dose-response curves are however rarely carried out on a proteome-wide scale due to the prohibitively long acquisition times usually required by bottom-up proteomic approaches using liquid chromatography coupled to mass spectrometry.

In this study, microflow liquid chromatography coupled to SWATH acquisition was used for rapid and robust proteome-wide dose-response drug profiling. The 45 min gradient setup enables exactly a one-hour run time injection-to-injection. The microflow-LC SWATH analysis of the LNCaP prostate cancer cell lines resulted in the identification of approximately 5'000 proteins consistently across all the time series and drug treatment conditions. The time-dimension of the dataset allows assessment of the cellular accessibility of the drug and the kinetics of drug action on the proteome scale. The drug concentration dimension allows the assessment of the level of sensitivity/reactivity of the proteins to the various drug concentrations. The dose-response curves of several proteins showed significant IC50 difference between the sensitive LNCaP and the resistant LNCaP-abl cell lines, pinpointing potential cellular pathways involved in the drug resistance mechanisms of LNCaP-abl.

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Mapping the Liver Interactome

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Protein-protein interactions (PPI) are a core dimension in biology as few proteins function in isolation. Recently, we and others have advanced the innovative protein correlation profiling (PCP) method for the large scale analysis of protein-protein interactions. In our PCP analysis we have combined data from mouse liver lysate separations using state-of-the-art chromatographic columns for either size exclusion (SEC), strong anion exchange (SAX), or hydrophobic interaction chromatography (HIC). In addition, we have used comprehensive proteome abundance measurements from both liver lysate subcellular fractionation experiments and cell-type specific proteome datasets.

Our data analysis pipeline uses binary comparison scores between each protein profile within each separation including Pearson correlation, cross-correlation and co-apex scores. These scores are used as features for each binary protein pair in our subsequent random forest machine learning approach. From this analysis we selected 116680 binary interactions with a score greater than 0.5 from the machine learning predictor. These binary pairs were integrated into a non-redundant network using the Clustering with Overlapping Neighbourhood Expansion (ClusterOne) package, while optimising the software settings for best precision and recall. This analysis identified 579 distinct liver protein complexes using stringent settings.

This dataset is quite novel as many of the detected protein complexes contain proteins that are only expressed in liver cell types. These interactions have therefore escaped detection in previous large-scale analyses of protein-protein interactions, which have focused on cultured cancer cell types. Follow-up experiments using immunoprecipitation-MS analysis of individual protein complexes is being used to confirm several of the novel interactions observed in liver tissues. The mouse liver interactome reported here will be extremely valuable for future experiments that examine how protein-protein interactions change after a perturbation such as either metabolic stress, or drug treatment.

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Investigating protein-protein interactions by crosslinking mass spectrometry

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Crosslinking mass spectrometry (XL-MS) is a technique for identifying protein-protein interactions. It can be applied *in vitro* and *in vivo*. It uses chemically reactive, MS-cleavable crosslinkers to covalently link interacting proteins. Proceeding digestion and enrichment, crosslinked peptides are then subjected to LC-MS/MS. The major advantage of MS-cleavable crosslinkers is their capacity to be fragmented, yielding two peptides. On further fragmentation identification of the two peptides, and hence crosslinked proteins, is achieved.

To explore the requirements of XL-MS for investigating protein-protein interactions, two current XL-MS methods were compared. The first, from the lab of Albert Heck, which uses DSSO and XlinkX (Lui, 2015) and second the lab of Andrea Sinz using BuUrBu and MeroX (Arlt, 2015). Three aspects of each method were compared: fragmentation, coverage and data analysis. Subsequently, three experiments were undertaken. Firstly, fragmentation analysis at CID 10-70eV using two peptides of known sequence was performed. Secondly, crosslinking analysis was undertaken using two proteins known to interact, and thirdly a complex mixture of 100-300 proteins.

Results from the first experiments indicate relative fragmentation of the crosslinker preferentially occurs and starts at 25 eV, whereas relative peptide backbone fragmentation does not peak until 30-35 eV, depending on the peptide. These results were then applied to subsequent experiments. Two proteins, Npl3 and Hmt1, were successfully crosslinked, with greater than 100 crosslinks identified. A mixture of yeast proteins were then crosslinked and analysed by MeroX and XlinkX. This resulted in 109 and 30 crosslinks being identified, respectively.

Both methods successfully identified crosslinked proteins, however, the number and type of crosslinks discovered differed between the techniques. MeroX identifies inter-, intra- and dead-end crosslinks between serine, threonine and lysine, whereas XlinkX identifies inter-peptide links between lysines. Our results indicate that different scales of XL-MS experiments will influence the choice of crosslinker and analysis software.

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Dissecting the subcellular secretory glycoproteome

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One of the most critical post-translational modifications in proteins is *N*-glycosylation, influencing the folding and function of ~ 1/3rd of the cellular proteome. *N*-glycosylation occurs when a preassembled glycan is transferred en bloc to a protein acceptor at specific protein sites (sequons) by the oligosaccharyltransferase. These glycans are then modified as proteins traverse the secretory pathway. Alterations in glycan structure and occupancy at specific sequons have significant clinical and industrial impact, leading to a growing number of diseases (Congenital Disorders of Glycosylation, cancer, etc) and to changes in secretion, half-life, and activity of proteins with biotechnological relevance. Further, glycans influence protein folding, stability, and traffic, and all of these depend on quality control processes that use glycans to decide on a protein's fate. It is therefore important to understand how glycosylation alterations occur and what is the impact of these changes in mature and immature protein expression. A more complete understanding of the causes and consequences of changes in *N*-glycosylation will lead to the design of better therapeutics, diagnostic strategies, and industrial bioprocesses.

To more effectively and efficiently study the mechanism of *N*-glycosylation and its physiological impact throughout the secretory pathway, we combined biochemical subcellular fractionation methods with quantitative SWATH-MS glycoproteomic workflows to measure site-specific and global changes in glycan occupancy and structure. We tested these methods in yeast cells with normal or altered glycan biosynthetic or quality control processes. We expanded these studies to look at the entire proteome and at the quantitative influence of protein quality control on protein maturation. Our protocols allow for rapid relative quantitative and qualitative proteomics and glycoproteomics of a variety of samples. Our methods and results have important implications in the understanding of the fundamentals of the glycosylation and quality control processes and on the industrial and medical applications of glyco-biotechnology.

1. Zacchi, L.F., Schulz, B.L. (2016). SWATH-MS glycoproteomics reveals consequences of defects in the glycosylation machinery. Molecular and Cellular Proteomics. Jul:15(7): 2435-47.

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Using proteomic analysis to uncover the mechanisms of non-protein amino acids attributed to neurological diseases

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Background: Neurodegenerative diseases such as Parkinson's disease and motor neurone disease are characterised by protein misfolding and deposition of protein aggregates in the nervous system. In 90% of cases these diseases are sporadic with no known cause. It is essential therefore that we identify the environmental factors involved. Non-protein amino acids (NPAAs) present in the environment have been implicated as causative factors in neurological disorders since they can be mis-incorporated into proteins in place of protein amino acids modifying native-protein structure generating misfolded aggregate-prone proteins.

Methods: We use quantitative proteomic approaches to examine the expression of proteins in human neuroblastoma cells (SH-SY5Y) after exposure to NPAAs to determine the mechanisms underlying their toxicity.

Results: Quantitative TMT labelling of proteins from human neuroblastoma cells treated with BMAA, a NPAA produced by cyanobacteria (blue-green algae), generated a comprehensive data set of differentially expressed proteins. Analysis revealed a proteotoxic stress response consistent with protein misfolding as well as changes to many pathways known to be involved in neurodegenerative diseases. Incorporation of certain NPAAs into proteins also resulted in a loss of solubility of specific proteins such as histone H4. In order to detect NPAAs in proteins with a greater sensitivity and specificity we developed a new method that allows NPAAs to be detected in peptides using mass spectrometry. This novel approach overcomes many limitations associated with protein hydrolysis which is currently the only method available.

Conclusions: The data presented supports the hypothesis that NPAAs can be misincorporated into proteins, cause protein misfolding and can impact on pathways implicated in neurodegenerative diseases.

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Variable selection and biological characterisation of chronic wounds using wound fluid proteomics and mass spectrometry

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Non-healing wounds are a significant problem for patients and healthcare systems worldwide. The underlying biochemistry, which drives non-healing outcomes in self-perpetuating leg wounds, is poorly understood. To address this knowledge deficit, a study of the proteins that compose the fluid, which exudates from these wounds, may provide important insight regarding treatment

response and healing outcome for patients. In this respect, we have conducted a clinical study that included the collection of biological samples and clinical / psychosocial data over a 24 week period, during which time patients received best-practice care. Biological samples were analysed using both data dependent and data independent acquisition mass spectrometry to detect and quantify the protein complement of the wound fluid. The resulting data were integrated with clinical measurements and contextualized by gene ontology annotations to enable deeper insight into the dynamic biological processes taking place within non-healing wounds. This identified key biological processes that may indicate specific underlying issues for a sub-set of wounds and their recalcitrant nature towards clinical care. A suite of biological markers that are indicative of wound healing outcome were also derived from these analyses and through the use of a novel regression algorithm. Unravelling the complex biology of non-healing wounds through proteome and clinical data integration provides some insight into the mechanisms associated with a patient's adverse or positive responses to clinical care. Such information can be developed further to inform clinical practices and enable the meaningful personalisation of wound management.

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How low can you go? The relationship between sample loading and protein quantification in SWATH-MS

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SWATH-MS is a powerful mass spectrometry technique with the potential to become a clinical tool for biomarker discovery and diagnostics. However, clinical tissue samples can be prohibitively limited, with a biopsy as small as 1 mg of tissue. This small amount of tissue necessarily dictates a lower loading on a mass spectrometer, but the effect on protein and peptide quantification is unclear. Therefore, the relationship between sample loading and protein and peptide quantification was investigated. A large batch of rat brain was digested and pooled to form a stock digest. This digest was loaded on to a Sciex 6600 TripleTOF at three concentrations: low, medium and high; with 6 replicates per group. The SWATH-MS peaks were extracted in PeakView with MS/MS^{ALL} SWATH Acquisition with a moderate spectral reference library (~4,300 proteins with 5% FDR). Quantified peptides were analysed in R and R/Bioconductor using a 1% FDR with an additional requirement to be present in all 6 samples. There was a moderate gain in the number of proteins and peptides quantified from the low to the medium loading groups. However, there was a modest gain in the moderate to high groups. These results indicate a small compromise in quantified proteins when loading samples from limited sources, such as biopsies.

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Combining metabolomics and genomics to dissect rice quality, and provide robust and trait-relevant tools to rice breeders.

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Aromatic rice commands the highest prices in both domestic and international markets because consumers prize both the mouth-watering aroma and delicate flavour of the rice. The major aromatic compound in fragrant rice is 2- acetyl 1-pyrroline (2AP). Using a panel of 380 diverse varieties of rice, metabolomics profiling of volatile compounds from the grain, and genome wide association with 33000 single nucleotide polymorphisms (SNPs), the objectives of this study were to identify (i) sensory traits that describe jasmine rice; (ii) the volatile compounds that define those sensory traits, and (iii) genetic markers for those compounds. The sensory descriptors fell into three clusters, with Cluster 1 describing high quality jasmine rice, Cluster 3 describing non-fragrant rice, and lower quality jasmine rices falling between the descriptors in Clusters 1 and 2. The compounds that most strongly discriminated the high quality jasmine rices from the other samples were 2AP and four other compounds, two of which required high resolution platforms to reveal their molecular structure and annotation. These five compounds associate with the same SNP on chromosome 8, several are fragrant with a low odour threshold, and they provide new information about the pathway of 2AP synthesis. Three QTL were found that associate with high or low amounts of the five compounds. Another 20 metabolites associated either positively or negatively with high quality jasmine fragrance. Significant genetic associations could be found for some of these compounds. By combining these platforms, we deliver new and valuable tools to breeders for selecting highly fragrant rice. We also deliver information and germplasm for the development of new populations targeted to provide appropriate phenotype data to identify QTLs for the other important metabolites identified here.

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MS-guided plant breeding

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Gluten is a diverse class of proteins found in wheat, rye, barley and oats. Coeliac disease (CD) affects ~70 million people globally. When CD patients ingest gluten, it triggers an inappropriate auto-immune reaction resulting in intestinal inflammation and damage. The only current treatment for CD and gluten intolerants is lifelong avoidance of dietary gluten, however, such diets are

costly and often low in fibre and high in calories, which in themselves are health risks. The worldwide market for gluten-free products is predicted to grow by ~25% over the next five years (to over US$7 billion). Gluten-free foods are commonplace, however, current methodologies (ELISA) do not accurately measure gluten as the antibodies are non-specific and show cross-reactivity.

We have developed a novel ultra-low gluten (ULG) barley variety in which the hordein (gluten) content was reduced to below 5 ppm. This was achieved using traditional breeding strategies to combine three recessive alleles, which act independently of each other to lower the hordein content in the parental varieties. We have employed advanced proteomics analysis to select the lines which showed the lowest gluten content and validate the low gluten content of the finished product.

Two LC-MS/MS approaches employing multiple reaction monitoring (MRM) and a data-independent acquisition strategy (SWATH) were used to quantify the complex protein mixtures present in nine barley varieties ranging from wild-type (gluten-containing) to ULG barley (gluten-free, < 20 ppm). Gluten-enriched and total protein fractions, extracted from flour using optimised protocols, was subjected to trypsin digestion by filter-aided sample preparation. The gluten peptide fragments were identified by high resolution LC-MS/MS with proteins identified from the *Poaceae* subset of proteins from Uniprot-KB. An MRM-based approach was explored for specific protein quantification and the results compared to those generated using variable window SWATH-MS.

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Towards the complete PTM map of CSLF6

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Mixed linkage (1,3;1,4)-β-glucan (MLG) is a major non-cellulosic polysaccharide of the commelinid monocot cell wall and an important soluble dietary fibre component found in abundance in cereal grains. Despite its importance, relatively little is known about the molecular mechanisms involved in the synthesis and assembly of this polysaccharide. Using functional genomics, the commelinid-specific Cellulose Synthase-Like (CSL) F, CSLH and CSLJ multi-gene families within the larger CAZy GT2 family have been identified as encoding the catalytic components of the MLG synthase enzyme (1, 2). The cellulose synthase-like F6 (CSLF6) protein is responsible for the majority of MLG present in the grasses, yet little is known about its regulation. For this purpose we are using a *Lolium multiflorum* (Italian rye grass) cell suspension culture system that makes abundant MLG in its walls (approx. 20-30%) to enrich the CSLF6 protein and identify the post-translational modifications (PTMs) present. The modifications included in this study are S-acylation, phosphorylation and disulphide bonding. With improved knowledge of the PTMs present on CSLF6 we can gain insights into its regulation and the production of MLG.

1. 1. Burton et al. (2006) Science 311, 1940 - 1942
2. 2. Doblin et al. (2009) PNAS USA 106, 5996 - 6001

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Next generation proteomics to detect biomarkers of pain and inflammation.

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Cattle are routinely subjected to painful surgical husbandry procedures, such as castration and dehorning. Quantifying the effectiveness of pain relief interventions during these procedures is challenging, due to the subjectivity and complexity of pain perception in animals and the inherent tendency for prey species to suppress their behavioural responses. Therefore, there are obvious limitations in the use of behavioural observations and routine biochemical or immunological assays restricted to individual targets (e.g. plasma cortisol) for detecting and quantifying the response to pain and stress. One approach, increasingly used in human biomedicine, is to develop an array of plasma biomarkers, which collectively respond to a stimulus. Next generation mass spectrometry techniques, such as SWATH-MS, can be applied to quantitative profiling of proteins (proteomics), lipids (lipidomics) and metabolites (metabolomics) in an unbiased manner and enable simultaneous evaluation of hundreds to thousands of various markers in virtually unlimited number of samples and thus provide more holistic representation of the physiological change. SWATH-MS analysis requires one off construction of spectral libraries which can be expanded as the project develops and shared between laboratories. In this study, SWATH-MS approach has been applied to monitor proteins that form a part of the systemic response to pain and inflammation and are putative targets of analgesic drugs. We describe the development of the foundational data and tools that do not only permit more thorough understanding of pain and inflammation in cattle but also have a potential to advance veterinary medicine in a manner that has not been possible before.

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Developmental proteomics: unravelling age specific differences in the human proteome

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Proteomics studies utilizing human plasma have to date focused largely on specific disease settings, on detecting the highest number of proteins or on the effect of certain drugs on changes in plasma protein expression. In addition, the majority of such studies have focused on adults, with limited number of studies and hence limited knowledge of the plasma proteome in infants and children.

HUPO Plasma Proteome Project recognizes the importance of analyzing and understanding age related differences in the plasma proteome by identifying this as one of their scientific aims and research priorities. In addition, the recently established Paediatric Proteomics (PediOme) initiative, a part of HUPO, aims to advance the use of proteomics techniques to solve major issues in paediatric medicine through characterization of the paediatric proteome across a wide-variety of tissues and biological samples.

Developmental Proteomics is a new concept that focuses on age specific differences in the human proteome. This new biology will be described through the use of SWATH-MS data independent acquisition as a new technology that is particularly suitable for developmental proteomics studies. Specifically, SWATH-MS allows confident identification of peptides over a dynamic range of 4 orders of magnitude, making it a useful tool for the analysis of biological specimens such as plasma. The use of SWATH-MS in neonatal and paediatric plasma samples to determine variability and subsequent comparison to adult plasma remains an exciting research opportunity, and one that has, to date, not been explored.

This presentation will outline the results of a recent study, which utilised SWATH-MS to profile the expression of plasma proteins associated with healthy human development.

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Coupling of gene-editing and proteomics to dissect the assembly and function of human mitochondrial complex I

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Complex I (NADH:ubiquinone oxidoreductase) is the first enzyme of the mitochondrial respiratory chain and is composed of 45 subunits in humans, making it one of the largest known multi-subunit membrane protein complexes. Complex I exists in supercomplex forms with respiratory chain complexes III and IV, which are together required for the generation of a transmembrane proton gradient used for the synthesis of ATP. Complex I is also a major source of damaging reactive oxygen species and its dysfunction is associated with mitochondrial disease, Parkinson's disease and ageing. Bacterial and human complex I share 14 core subunits that are essential for enzymatic function; however, the roles and necessity of the remaining 31 human accessory subunits is unclear. The incorporation of accessory subunits into the complex increases the cellular energetic cost and has necessitated the involvement of numerous assembly factors for complex I biogenesis.

We used gene-editing technology to generate human knockout cell lines for each accessory subunit. We show that 25 subunits are strictly required for assembly of a functional complex, and one subunit is essential for cell viability. Comprehensive quantitative proteomic analysis of all cell lines revealed that loss of each subunit affects the stability of other subunits residing in the same structural module. Analysis of proteomic changes after the loss of specific modules revealed two new assembly factors required for building the distal portion of the complex I membrane arm. Our results demonstrate the broad importance of accessory subunits in the structure and function of human complex I.

Coupling gene-editing technology with large scale proteomics represents a powerful tool for dissecting multisubunit complexes and enabling the study of complex dysfunction at a cellular level.

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Quantification of the exercise-regulated peptidome and the chemical modification of bioactive peptides

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The plasma peptidome contains the entire complement of low molecular weight endogenous peptides derived from secretion, protease activity and post-translational modifications and is a rich source of biomarkers and novel bioactives. To determine the utility of peptidomics we examined the effects of exercise on the plasma peptidome because it conveys many of its physiological benefits via the blood stream. We compared methods to rapidly isolate the plasma peptidome revealing trichloroacetic acetic precipitation under denaturing conditions combined with mixed-mode solid-phase extraction produces the greatest depth. We applied this approach to the multiplexed temporal quantification of the exercise-regulated plasma peptidome combined with 2D-LC-MS/MS. Peptides were analysed with multiple fragmentation methods including HCD and EThcD which resulted in the quantification of 5,548 unique endogenous peptides. The plasma peptidome underwent dynamic changes during exercise on a time-scale of minutes and this was rapidly reversible following exercise cessation. Among acutely regulated peptides were many known hormones including insulin, glucagon, ghrelin, bradykinin, cholecystokinin and secretogranins. Using site-specific protease mapping to known substrates and subsequent enrichment analysis, we generated a protease:substrate network to infer the activity of proteases regulated with exercise. Our analysis also included the characterisation of PTMs such as amidation and glycosylation. The utility of multiple fragmentation methods were able localise N-glycan modification sites in the glycopeptidome. These data reveal the utility of peptidomic quantification to temporally quantify bioactive peptides, biomarkers, PTMs and proteolytic activity during pathophysiological processes.

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Application of metabolomics and proteomics for biomarker discovery and development of therapies for the transmissible cancer, Tasmanian Devil Facial Tumour Disease

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The wild population of Tasmanian devils has declined by more than 80% due to the fatal Devil Facial Tumour Disease (DFTD), one of the few known naturally-occurring, clonally transmissible cancers. There is currently no pre-clinical test or effective treatment for this disease that threatens the species with extinction. Our major goals are to develop a diagnostic test to detect the disease at the latent (pre-clinical) stage and to evaluate immunotherapeutic drugs, such as the approved topical agent imiquimod (R-837), as potential treatments for this aggressive cancer.

Biomarker discovery for DFTD was performed using non-targeted metabolomics analysis of serum samples collected from wild Tasmanian devils (35 controls and 35 with tumours) by HPLC/MS. Multivariate models (PLS/DA, random forests) were used to identify potential discriminating features in an age-matched training set. Positive markers for DFTD included amino acids, metabolites related to lipid absorption and a series of overlapping fibrinogen peptides, while cortisol and urea were the most significant health predictors. A support vector machine model utilizing only the major peptide and seven other metabolites was able to classify samples with 94% sensitivity and specificity.

Label-free shotgun proteomics was used to study the effects of imiquimod on DFTD tumour cells. Imiquimod is a toll-like receptor 7 (TLR7) agonist that in humans induces immune cell infiltration and cytotoxic immune pathways. Imiquimod may augment this response by direct activation of tumour cell apoptosis, but the underlying mechanism is unknown. Many of the proteins altered by imiquimod were related to ER stress, cell cycle arrest and increased antioxidant activity. Unlike DFTD tumour cells, imiquimod-treated Devil fibroblasts (TLR7-) lacked the hallmarks of ER stress and did not undergo apoptosis. Targeted induction of ER stress is therefore one potential mechanism for the selective effects of imiquimod on DFTD cells and ER stress pathways represent novel targets for DFTD treatment.

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Proteome analysis of endometriosis patient tissues leads to development of screening assays in sera

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Endometriosis is a condition where the uterine inner layer, endometrium, is found in pelvic cavity and/or in ovaries or on the surface of bladder or rectum. The prevalence of disease in women in their reproductive age is 2-10%, and up to 50% in women with unsolved infertility and/or pain symptoms. Women with endometriosis suffer from chronic pelvic pain caused by innervation and inflammation at locations of endometriotic lesions. Diagnosis is an invasive procedure and treatments are not always effective or bring up severe side effects and, therefore, new information on possible therapeutic targets and diagnosis for endometriosis are essential. The complexity of different types of endometriosis as well as heterogeneous nature of endometrium tissue along its modulation with menstrual cycle makes disease difficult to study in the lab.

The work described in the lecture discusses how a phased mass spectrometry-based approach for discovery, screening, and validation of protein biomarkers with diagnostic value was followed. Technically the workflow was optimized in terms of sample sensitivity and robustness, allowing quantification of several 1000 proteins with a technical CV of 10 % in 10s of patient tissue types. For quantitative analysis, several different approaches were studied including five statistical methods that were evaluated for use with label-free quantitative proteomics data.

Initially we characterized and reported high quality SRM transitions for 168 disease markers, that were then subsequently screened and reduced to a single assay containing 93 synthetic peptides that are currently being used in a multiplexed SRM and SWATH analysis in 100 serum samples from patients. Methods established, study setup and our most recent findings on our MS analysis will be reported.

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Towards Personalised Medicine: The Road Ahead

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Following on from the completion of the human genome in 2003, the emergence of a comprehensive Omics pipeline, comprising multiple orthogonal platforms encompassing genomics, proteomics, metabolomics, transcriptomics and interactomics, has brought with it exponentially increasing volumes of experimental data arising from the use of a number of high throughput data-intensive technologies (e.g. mass spectrometry, microarray, NextGen sequencing) that have recently been developed. Whilst these data hold the key to an improved understanding of human health and disease, and should support the emerging field of personalized medicine, effective and efficient mining of this data is not without its trials and tribulations, including how to handle

the complex information involved, how to integrate the data from a significant number of very heterogeneous platforms, what principles and standards must be adopted to ensure the veracity of the findings as well as potential ethical and funding issues.

In this presentation I will overview the Omics pipeline, present the concept of personalized medicine, discuss some of the problems that could arise in the handling of the "Big Data" generated in the road ahead and the role the human proteome organization (HUPO) has in addressing some of these problems.

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Chemical proteomics based target identification of bioactive small molecules

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Exploring the interaction of a small molecule with its target protein is pivotal to understand the cellular mechanism by which the small molecule acts. However, discovering the on- or off-target proteins of small molecule is often the most challenging and time-consuming step. Chemical proteomics has played as a key research engine to identify direct interacting proteins and to explore mechanisms of action of small molecules towards functional and translational applications. A number of methodologies including conventional affinity chromatography using labeled small molecules as well as recent target identification methods with label-free small molecules such as Drug Affinity Responsive Target Stability (DARTS), Stability of Proteins from Rates of Oxidation (SPROX), Cellular Thermal Shift Assay (CETSA), and Thermal Proteome Profiling (TPP) have been developed and applied to identify the direct binding proteins of small molecules. This interaction information of small molecule and target protein facilitates structure based better drug development and functional annotation of target protein as well. Furthermore, integration of MALDI-MS imaging technology with chemical proteomics will enable to validate the interaction of label-free small molecule with target protein in tissue that harnesses the preclinical studies of small molecules in respect with their efficacy, toxicity, and pharmacokinetics. In this presentation, recent advances of chemical proteomics for target identification of small molecules towards functional and translational applications will be presented by introducing our case studies.

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Finding cancer fats through integrated proteomics and lipidomics

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Obesity, elevated cholesterol and triglyceride levels are associated with cancer progression and recurrence. However, the specific lipid metabolism pathways involved remain mostly unclear. We combined mass spectrometry-based lipidomics and proteomics in cell line models to determine lipid metabolism differences between oesophageal adenocarcinoma and its pre-malignant condition, Barrett's oesophagus. Untargeted lipidomics in 5 cell lines (5 replicates each) measured 6843 features, of which 901 features with p-value <0.001 and fold change >2 were selected for MS/MS using a range of collision energies. Spectra were matched in MS-DIAL against the FiehnRT Lipid database (v16), confidently identifying 92 lipids. Strikingly, the results suggest that oesophageal adenocarcinoma cancer is associated with changes in 3 lipid metabolism pathways. For 2 of these pathways, corresponding lipid metabolic enzyme changes were detected in the proteomics analysis of the same cell lysates. The relevance of these lipid metabolic pathway changes were confirmed in patient biopsy samples using targeted MRM-MS assays.

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Ion mobility spectrometry for enhanced omic analyses

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Mass spectrometry (MS)-based technologies are playing a growing role in the analysis of complex samples. Despite significant advances in MS technology, currently it is difficult to obtain measurements of both high throughput and high sensitivity for samples with great dynamic ranges such as plasma and serum. This problem ultimately results in the inability to effectively account for variation among sample conditions and/or biodiversity leading to inconsequential findings for samples which have great variation. To address this challenge, we have coupled an ion mobility separation (IMS) with MS to afford greatly improved measurement throughput, sensitivity, robustness, and quantitative capabilities for the rapid analysis of complex samples. The benefits we have observed in omic studies with IMS-MS will be summarized in this presentation.

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Comprehensive analysis of polyunsaturated fatty acid-derived lipid mediators in human plasma pre- and post-ingestion of aspirin: A pilot study

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Chronic inflammation is associated with many diseases including rheumatoid arthritis, asthma, diabetes, inflammatory bowel disease and neurological diseases such as Alzheimer's disease (Serhan, 2014). Lipid mediators, derived from the oxygenation of polyunsaturated fatty acids (PUFAs) through enzymatic or free-radical mediated mechanisms, are implicated in the promotion (pro-inflammatory) and resolution (pro-resolving) of inflammation. In addition, it is known that low-dose aspirin (acetylsalicylic acid) inhibits excessive inflammation by significantly reducing the levels of pro-inflammatory lipid mediators and increasing the synthesis of the specialized pro-resolving mediators (SPM). At the present time, however, the physiological effect of a single aspirin dose on plasma pro-resolving lipid mediator levels in healthy human subjects, or on therapeutic response in intensive care patients, is unclear. Here, we will describe the development of optimized collection, storage, extraction and liquid chromatography–tandem mass spectrometry analysis conditions for the clinical measurement of plasma lipid mediators, in response to aspirin in (i) a pilot study of healthy volunteers with the hope of extending this analysis to (ii) a randomized double blind placebo control study to evaluate the effect of aspirin on clinical outcome in intensive care patients.

1. Serhan CN (2014) Pro-resolving lipid mediators are leads for resolution physiology. Nat, 510, 92-101.

54

Mass Spectrometry: a platform for personalised medicine

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There is immense potential for novel molecular technology to revolutionise medical care. In this presentation Mr Babar Vaqas, Neurosurgeon from the Takats lab at Imperial College London, will outline the many facets of surgical mass spectrometry and its capacity to influence the surgical decisions of the future. Mr Vaqas will focus on the difficulties encountered when operating and treating brain cancer, and how the advent of personalised medicine may provide the badly needed solutions needed for patients who may not survive longer than a few months.

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Tissue homogenization with the picosecond infrared laser for proteomics

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Homogenization is a critical step in the analysis of proteomes of tissues. With many classical homogenization methods not all parts of the tissue are completely dispersed. As a result after centrifugation an insoluble pellet remains, containing proteins, which are lost for the analysis. A problem occurring in each kind of homogenization method is associated with release of enzymes changing the composition of the proteomes. The picosecond infrared laser (PIRL) is promising to improve the quality of tissue homogenates. During irradiation water molecules in the tissue absorb the energy of the laser. Because of the picosecond pulse the absorbed energy of the water molecules is nearly completely transformed into translational energy. Thus during irradiation with PIRL the tissue is ablated by cold vaporization.

In this study by applying diverse proteomic approaches PIRL homogenates were analyzed and compared with homogenates yielded by mechanical homogenization. Comparison of PIRL tissue homogenates with mechanical tissue homogenates by bottom-up proteomics revealed a significant higher yield of the number of identified proteins. Analysis of the different homogenates with two-dimensional electrophoresis showed a larger number of protein species in the case of the PIRL homogenization. The mechanical homogenate was characterized by a larger number of proteolytic degradation products. The lower degree of proteolytic degradation in the PIRL homogenate presumably can be explained by the ultrafast process of the conversion of intact tissue into the frozen condensate of the tissue aerosol.

In conclusion homogenization of tissues with PIRL is advantageous because the dispersion of tissues is more complete, the homogenate is more homogeneous, it is faster and very soft. Furthermore cold vaporization of tissue with PIRL is giving higher yields in the total amount of proteins, in the number of identified proteins and in the number of protein species (proteoforms).

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Large Scale Analytics of Metabolite Annotations from Imaging Mass Spectrometry

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Imaging Mass Spectrometry (MS) is a maturing technology progressing towards being a routine analytical tool, partially driven by the uptake of high resolving power instrumentation, particularly for small molecule analysis where imaging MS looks set to unlock another dimension in metabolomics. Consequently, more high-quality data is being produced from larger scale studies with significant cohort sizes. To begin bridging the gap between spectra and biological information we have recently developed a high-throughput platform for false-discovery-rate (FDR) controlled molecular annotation of imaging MS. We are now performing inter-study analytics to address some essential questions in spatial metabolomics.

We recruited members of the imaging mass spectrometry community recruitment to submit datasets to our open-access online annotation platform along with some essential sample metadata Every datasets was submitted to our bioinformatic pipeline deployed using big-data technology for high-throughput FDR controlled molecular annotation. All datasets were searched against the Human Metabolomics DataBase for [+H+, +Na+, +K+, or -H-, +Cl-] adducts in positive and negative mode respectively and

annotations reported at an estimated FDR of 0.1 to enable comparison. To mine the thousands of annotations produced we leveraged big-data analytical tools designed for business intelligence of large heterogeneous information streams.

Over 20 laboratories provided more than 300 datasets for annotation (an estimated raw data volume of >60TB). The annotation database includes 18 species, some with multiple disease states, all HR mass analysers, DESI, MALDI and IR-MALDESI ionisation sources. Approximately 6000 unique molecular formulas were annotated across all datasets with a median of ~300 per data-set Untargeted analysis gave us a holistic view over all datasets, enabling us to investigate which molecules are indicative of technology, organism, or tissue type. Alongside high-level queries, comparative analysis permits 'biomarker discovery' approaches immediately enhanced with additional information on which other systems discriminative molecules were detected in.

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When proteomics meets lipidomics

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The separate disciplines of proteomics and lipidomics are now well-established and have evolved independently over the last two decades. In parallel, over the same time period, native mass spectrometry experiments have advanced such that survival of intact protein complexes in the gas phase is now unequivocal. A decade later, with the ability to observe intact membrane protein complexes, it became apparent that these assemblies were often extracted with their associated lipid environments intact. Consequently, it is now possible to study lipids that interact directly with proteins rather than collections of proteins or lipids reported in separate proteomic or lipidomic investigations.

During this lecture I will show how the combination of proteomics and lipidomics is enabling us to identify lipids that are critical to the structure and function of membrane proteins. During our research we have also discovered lipids that modulate associations between membrane proteins, remodel the lipid bilayer in the vicinity of complexes and compete for drug binding sites on receptors. I will use a range of membrane protein assemblies to illustrate these lipid connections including ion channels, transporters, membrane embedded motors and GPCRs.

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Development of a novel encyclopaedic peptide spectral library using the liquid fraction of sheep blood

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Background: Identifying protein biomarkers helps in defining and predicting animal response to illness. We have developed a platform capable of detecting acute phase protein (APP) inflammation markers and their alterations in the liquid fraction of sheep blood using SWATH-MS. This approach is an attractive alternative to antibody ELISA technology which can be particularly costly and time consuming. A broader tool to measure plasma or serum levels of many different proteins using proteomics would be highly advantageous. It was hypothesised that every injury is accompanied by proteomic alterations in specific biomarkers. The main research objectives were to develop a feasible proteomic method to characterise the ovine acellular circulating proteome and apply it to samples from healthy and ill sheep to potentially detect candidate markers of inflammation.

Method: A novel encyclopaedic peptide spectral library was constructed from several hundred samples derived from the ovine acellular circulating proteome of sick and healthy sheep using a TripleTOF® 5600 instrument by shotgun proteomics. SWATH data extraction strategy was used alongside the library on the same platform to interrogate samples of an ovine model of intensive care in which the subjects were exposed to acute sepsis and inflammation.

Results: Over 700 protein alterations were detected, verified and quantitated between normal and endotoxaemic sheep plasma. It was confirmed that the subjects of the sheep model of intensive care study were phenotypically non-identical. Apart from over 50 APP, at least 75 other proteins were potentially endotoxin-specific candidate markers of inflammation.

Conclusion: Using SWATH and a novel sheep-specific peptide spectral library to this scale for the first time enabled the identification of a colossal number of plasma proteins and their alterations during inflammation. The potential applications for this approach are in veterinary pathology, animal welfare and screening laboratory animals before inclusion in experimental groups to minimise differences, for example.

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Sample Replication Effects on Statistical Power in Multiplexed Immunoassay Analysis

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It is known that increasing replication via expanding the number of biological and or technical replicates analysed, increases overall experimental precision and statistical power. While the examples and data presented here will focus on the current multiplexed immunoassay experimental practice, where all samples are generally analysed in duplicate assays, the techniques presented are generic and apply to a wide variety of experimental designs and biological data types. The data presented is the fluorescence responses from screening for 14 analytes within 354 plasma samples, used in a clinical trial, collected from 177 patients and across 60 96-well plates. For plate or array based multiplexed immunoassay experiments the replicated design strategy halves the numbers of biological replicates that can be analysed per array or plate and therefore, essentially doubles the assay costs of the experiment. Here it's shown that when the level of technical variance is less than the biological variances that

for most types of immunoassay experiments, such as screening biological samples for the expression level of proteins, there is no benefit gained from using technical replicates when the number of biological replicates exceeds 20 samples. It is also presented that when the usual numbers of technical replicate assays, according to common practice, are replaced by unique biological replicate assays that the contribution to the observed assay variance from the biological variances decreases by 50%. Methods for determining the level of technical and biological variances in a sample are discussed, plus a new method is presented for determining the level of technical and biological variance within a sample.

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Cavitron ultrasonic surgical aspirates are novel sources of glioblastoma-derived extracellular vesicles

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Glioblastoma (GBM), or stage IV astrocytoma, is the most common and lethal primary brain tumour in adults. GBM carries an exceedingly poor prognosis due to its aggressive, invasive and recurrent nature. EVs are 30-1000 nm membranous vesicles released by all cells and contain molecules that reflect their cell of origin. Studies have showed that extracellular vesicles (EV) play key roles in GBM biology and represent novel biomarker reservoirs.

Recently, the Kaufman lab described the most comprehensive *in vitro* GBM-EV proteome signature to date [1]. EVs were isolated from six established GBM cell-lines and analysed by quantitative high resolution mass spectrometry. A total of 844 proteins were identified, of which 145 were common to the GBM-EV from all six cell-lines. Levels of 14 GBM-EV proteins significantly correlated to GBM cell invasiveness. We aim to translate this *in vitro* GBM-EV protein signature to clinical specimens in order to describe new biomarkers that are predictive of GBM progression and invasion.

We have identified and characterised Cavitron Ultrasonic Surgical Aspirate (CUSA) fluids as novel and rich sources of brain tumour-EVs, demonstrated by transmission electron microscopy (TEM), nanosight particle tracking analysis (NTA) and label-free quantitative mass spectrometry analysis. Quantitative proteomic analysis of enriched CUSA-EV from high-grade and low-grade gliomas showed that there was greater than 70% coverage of the *in vitro* GBM-EV proteome signature in CUSA-EV. In addition, levels of the proposed invasion-related markers were significantly higher in high-grade gliomas in comparison to lower-grade gliomas.

Reference:

Mallawaaratchy, D.M., Hallal S. et al., Comprehensive proteome profiling of glioblastoma-derived extracellular vesicles identifies markers for more aggressive disease. Journal of Neuro-Oncology, 2016: p. 1-12.

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Digestion-free Targeted LC-MS Quantification of Circulating Thymosin beta 4 in Heart Failure

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17. Christchurch Heart Institute, University of Otago, New Zealand, Otogo, New Zealand

Tryptic Digestion is a necessity for big proteins, to overcome signal dilution due to multiple charge envelope and wide isotopic distribution when doing quantification by MRM. For smaller proteins, avoidance of digestion step could preserve peptidoforms information, and minimise variability. Thymosin beta-4 (TB4) is an X-linked gene product with cardioprotective properties. TB4 is

a good candidate for developing a digestion-free LC-MS quantification, as it is not excessively big (approximately 5 kDa), very soluble, and is relatively abundant. We developed a digestion-free dilute-and-shoot method to quantify TB4 in plasma, then piloted the assay in heart failure (HF) cohort.

MRM for TB4 was developed empirically from synthetic standards, standard curve prepared by diluting in rabbit plasma. Plasma samples were spiked with heavy isotope internal standard, then "crashed" with acetonitrile. The assay was piloted in a nationwide heart failure cohort (n=438) with controls (n=219).

In HF patients compared to controls, plasma TB4 was significantly elevated [1265 (638–2146) ng/mL vs. 985 (421–1723) ng/mL, p=0.002]. Elevation seems to be primarily driven by women with heart failure with preserved ejection fraction (HFpEF) [1623(1040-2625) ng/ml]. Over the two years follow-up period, there were 60 deaths among patients with HF. Adjusted for NYHA class, N-terminal pro-B-type natriuretic peptide, age, and myocardial infarction, hazard ratio to all-cause mortality is significantly higher in women with elevated TB4 (1.668, p=0.036), but not in men (0.791, p=0.456) with HF. By also doing pairwise correlation of the TB4 measurements with biomarker information obtained from a multiplex proximity extension assay. We found that TB4 is strongly correlated (R > 0.7, p<<0.001) with a cluster of seven markers, six of which are either X-linked or regulated by sex-hormones. Given that the limited therapeutic options and poorly understood pathophysiology of HFpEF, our findings could lead to novel hypothesis.

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Generation of PCT SWATH-MS based proteomic profile of rat organs

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In diagnostic cancer pathology, the identification of specific proteins or combinations of proteins by immunohistochemistry is often essential to arrive at a correct diagnosis and to guide patient management. The goal of ProCan is to investigate the application of high-throughput tissue protein profiling to this process by a combination of Pressure Cycling Technology (PCT) and Sequential Window Acquisition of all Theoretical Spectra (SWATH) mass spectrometry (MS). PCT SWATH-MS has demonstrated applicability to very small tissue samples with results potentially available in a clinically applicable turn-around-time (1).

One of the major goals of ProCan is to quantify proteins in tumour samples obtained from a variety of organs. Current evidence indicates that tissue proteomes obtained from PCT- SWATH are clustered based on their site of origin. Developing a SWATH based profile of different normal tissues will provide an important reference to guide interpretation of cancer proteomic data. Specifically, it will assist in understanding aspects of tumour differentiation, and tissue of origin in the case of metastatic disease.

In preliminary work, we have applied PCT SWATH-MS to create a complete proteomic profile (specifically an ion library essential for SWATH analysis) of samples collected from six different rat organs, (brain, liver, muscle, spleen, lung and kidney). The detailed results will be presented and will be uploaded to a publically available database such as SWATHAtlas, making the data available to all researchers.

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Physical activity elevates circulating levels of the neuroprotective LG3 peptide: potential novel stroke therapy?

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Stroke is a leading cause of death and disability globally and in Australia. Interestingly, physical activity and exercise is thought to reduce stroke risk and enhance rehabilitation following stroke, although the mechanisms remain unclear. Perlecan is a major ECM protein of vascular basement membranes, neuromuscular junctions and cartilage, which is known to be proteolytically processed to release a C-terminal bioactive fragment known as the laminin-type G3 (LG3) peptide. Importantly, the LG3 peptide is naturally released in the stroke-injured brain and is profoundly neuroprotective / neuroreparative when administered systemically in animals. Our research team has found that the LG3 peptide was significantly increased in the urine of physically active mining workers compared to sedentary controls. However, no previous studies have investigated the relationship between physical activity and changes in LG3 abundance in human serum where it might have therapeutic benefit. Therefore, the aim of this study was to measure the serum levels of LG3 fragments in response to exercise. Venous blood samples were collected at pre- and post-exercise from six well-trained male athletes who had participated in an intermittent, high-intensity trial (85% maximum aerobic capacity, VO_2 max) and a continuous, moderate-intensity trial (60% VO_2 max). Detection and quantification of LG3 was approached through the development of a Selected Reaction Monitoring (SRM) assay to specifically target LG3 peptide in serum. LG3 peptide levels significantly increased in response to moderate-intensity exercise (p = 0.026). The findings of this

study suggest that circulating levels of the neuroprotective / neuroreparative LG3 peptide can be significantly increased under normal physiological conditions in healthy individuals following moderate exercise. In the future the results of this study may help establish a baseline level of physical activity required to induce elevated levels of the LG3 peptide in the blood which may have clinical utility.

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The future of glycan MALDI mass spectrometry imaging

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MALDI mass spectrometry imaging of *N*-glycans was established 4 years ago and is now routinely used by 3 groups around the World. Since our group's proof-of-principle paper in 2015, we have published several other papers that spatially investigate the *N*-glycome of formalin-fixed paraffin-embedded (FFPE) tissues that have been extracted from osteoarthritis and ovarian cancer patients. However, further work is required by our group to *(i)* improve and optimize this workflow, and *(ii)* verify whether this technique can be used to discover potential diagnostic markers for early-stage diseases. This presentation will provide *(i)* detailed instructions on our established method for groups new to MALDI mass spectrometry imaging and *(ii)* a vision for the future of this new glycomic technology.

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4. Arun V. Everest-Dass, Matthew T. Briggs, Gurjeet Kaur, Martin K. Oehler, Peter Hoffmann, Nicolle H Packer: N -Glycan MALDI Imaging Mass Spectrometry on Formalin-Fixed Paraffin-Embedded Tissue Enables the Delineation of Ovarian Cancer Tissues. Molecular & Cellular Proteomics 07/2016; DOI:10.1074/mcp.M116.059816

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High throughput and accurate quantitation of phosphoproteomics for biological signaling

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The most accurate quantitation on protein complex mixtures can be accomplished by employing TMT SPS MS3 method on Tribrid mass spectrometers. However, this method is not optimal for phosphopeptide quantitation due to special characteristics in phosphopeptide fragmentation patterns. In this study, we developed methods to address this limitation and provide high phosphopeptide identifications and accurate quantitation.

Digested HeLa cells were labeled with TMT10plex™ reagents and mixed at ratios of 16:8:4:2:1:1:2:4:8:16. Yeast digest was labeled with the last 5 channels mixed equimolar (0:0:0:0:0:1:1:1:1:1) and spiked into TMT-labeled HeLa digest sample as interference. This resulted in Hela digests with first five channels that were free of interference and the last five channels interfered by yeast proteome. The mixed samples were further enriched for phosphopeptides and analyzed on Orbitrap Fusion and Lumos MS.

Ratio distortion was observed for phosphopeptide analysis on HeLa and yeast mixture, when using MS2 workflow. This was due to interfering ions co-isolated with precursor ions. The use of SPS MS3 improved quantitation accuracy. However there was 50% of loss in the number of phosphopeptides identified. The loss was due to the strong presence of the neutral loss peak specific to phosphopeptide, which limited the identifications from CID MS2 spectra. We thus developed and optimized two new MS3 instrument methods to reduce the loss of phosphopeptide identifications. This approach minimized loss to less than 30%, while maintaining quantitation accuracy benefits given by SPS MS3.

The new MS3 methods were applied to large scale phosphoproteome characterization in A549 cell line upon insulin and IGF-1 treatments. Overall, 3,378 protein groups and 12,465 phosphopeptides were identified of which 10,436 were quantifiable. The accurate and reproducible measurement enabled mapping regulated phosphorylation sites to numerous signaling pathways including mTOR signaling and AMPK signaling pathways.

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Characterising dynamic changes to MLKL during necroptotic cell death.

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Necroptosis is a form of programmed cell death that is activated by signalling from death receptor ligands, such as TNF, and Toll-like receptors. Initiation of necroptosis results in disruption of the inner plasma membrane, cell swelling and release of cellular components that promote inflammation. Key effector proteins in the pathway include receptor interacting protein kinase (RIPK)-1, RIPK3 and the pseudokinase, mixed-lineage kinase domain-like (MLKL). MLKL is the most terminal known component of the necroptotic pathway and contains two functional domains, a N-terminal four-helix bundle (4HB) and a C-terminal pseudokinase domain (PsKD) tethered together by a two-helix “brace” linker. MLKL has retained the ability to bind ATP in the PsKD despite having no catalytic kinase activity. MLKL is activated following phosphorylation of the PsKD by RIPK3, which is thought to induce conformational changes that release the 4HB domain. Subsequently, MLKL is known to oligomerise and translocate to the inner plasma membrane where it destabilises the membrane to promote cell death.

We employed biophysical techniques, including analytical ultracentifugation and size exclusion chromatography, to deduce whether the interaction with ATP regulates MLKL oligomer formation. To understand the structure and dynamics of MLKL activation, we utilized multiple proteomic techniques including hydrogen-deuterium exchange mass spectrometry (HDX-MS) and crosslinking mass spectrometry (XL-MS) to characterize the changes that occur to MLKL during activation and oligomerisation, and upon ATP binding. Our analysis provided insights into MLKL oligomerisation; a large reorganization of the two-helical “brace” region and protection of several regions in the 4HB and PsKD were observed. Together these results have revealed that MLKL undergoes extensive reorganisation upon activation and oligomerisation, with the changes induced by ATP binding likely reflecting the molecular switch function of MLKL’s pseudokinase domain in controlling cell death by necroptosis.

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A DIA-based Phosphoproteomic Study of Signalling Transduction via the Chemokine Receptor CCR2

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Inflammation is the body’s response to injury or infection. A hallmark of inflammation is the accumulation of leukocytes, which remove pathogens and necrotic tissue by phagocytosis and proteolytic degradation. Leukocytes are mainly recruited by chemokine activation of chemokine receptors, resulting in leukocyte morphological changes, extravasation into the inflamed tissue and chemotaxis along the chemokine gradient to the site of injury or infection. However, dysregulation of leukocytes/monocytes can result in inflammatory diseases. Therefore, chemokine receptors and chemokines are potential therapeutic targets in a wide range of inflammatory diseases.

CCL2 (or monocyte chemoattractant protein-1, MCP-1) and CCR2 is the major chemokine and receptor pair, involved in recruitment of monocytes, which subsequently differentiate into macrophages, contributing to the pathogenesis of atherosclerosis, obesity and type 2 diabetes. Considering the importance of CCL2-CCR2 in these diseases, there is strong motivation to understand their mechanism of activation and signalling. In particular, while CCR2 is known to signal via G protein and β-arrestin-mediated pathways, the downstream signalling pathways have not been thoroughly explored.

Protein phosphorylation and dephosphorylation are crucial for cellular signal transduction. Dynamic regulation of reversible, site-specific protein phosphorylation is critical to the signalling networks. Here, we exploited phosphoproteomic analyses in combination with cutting-edge data-independent acquisition (DIA)-mass spectrometry to unravel signal transduction events and their dynamic regulation in CCL2-activated CCR2-expressing cells. We created a library containing more than 25,000 phosphopeptides. During the first hour of CCL2-activation, we reproducibly and accurately quantified ~15,000 phosphopeptides in each repeat without any fractionation. From this study, we identified the key components and major pathways involved in CCR2 signalling.

Given the importance of CCL2-CCR2 signalling in the pathogenesis of many debilitating and prevalent diseases, this study does not only provide in-depth and novel insights into this signalling pathway, but will also guide the identification of potential therapeutic targets.

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Investigation of SOCS5 signalling complexes by mass spectrometry uncovers a role in breast cancer

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The Suppressor of Cytokine Signalling (SOCS) family of proteins are critical negative feedback inhibitors of cytokine and growth factor signalling, helping to restore homeostasis and prevent excessive pathological signalling. SOCS5 has been suggested to negatively regulate EGF receptor levels in mammalian cells and has been implicated in EGF receptor driven malignancies. However its precise physiological function and how it might act to regulate signalling pathways has remained poorly characterised. We have utilised mass spectrometry to define the SOCS5 interactome and have identified a number of distinct protein complexes. These analyses have also revealed that the SOCS5 N-terminal region, which is predicted to be largely unstructured and has no known function, to be heavily phosphorylated and we hypothesis that SOCS5 acts as a scaffold to support multiple signalling

complexes. Interestingly, many of the identified SOCS5 interacting proteins have been implicated as drivers of aberrant signalling pathways in human breast cancer. Strikingly, in the Polyoma Middle T antigen model of breast cancer, SOCS5-deficient mice exhibit accelerated tumour onset and growth relative to wild-type mice, thus providing the first *in vivo* evidence that mammalian SOCS5 can act to regulate tumorigenesis. Importantly, analysis of expression databases indicates that SOCS5 is down regulated in a majority of patients with invasive ductal breast carcinoma (TCGA dataset, top 7% under-expressed genes, p=2.53E-23) and may represent an important physiological regulator of breast cancer progression.

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PTMOracle: a Cytoscape app for co-visualising and co-analysing post-translational modifications in protein interaction networks

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Post-translational modifications of proteins (PTMs) act as key regulators of protein activity and of protein-protein interactions (PPIs). To date however, it has been difficult to comprehensively explore functional links between PTMs and PPIs. To address this, we developed PTMOracle: a Cytoscape app for co-analysing PTMs within PPI networks. For proteins of interest, or a whole proteome, PTMOracle can generate network visualisations to reveal complex PTM-associated relationships. PTMOracle also allows extensive data to be integrated and co-analysed with PPI networks, such as protein sequences, motifs and structural annotations such as domains and disordered regions. PTMOracle contains the OraclePainter for colouring proteins by modifications, OracleTools for network analytics, and OracleResults for exploring tabulated findings. To illustrate the use of PTMOracle, we investigated PTM-associated relationships and their role in PPIs in yeast. We explored a histone-associated network, showed how integrative approaches can be used to predict kinases involved in phosphodegrons. We also demonstrated how these approaches can be used to identify simple “decision-making modules”, which consists of a decision-making protein, two modifying enzymes and two protein interaction partners. Such modules are likely to be important for understanding how cellular signals are integrated, and how different biological outcomes are managed by multiple types of PTMs. The PTMOracle is open-source and available on the Cytoscape app store: http://apps.cytoscape.org/apps/ptmoracle.

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Exploring the phosphoproteome to understand the roles of p38 and IAPs in TNF signalling

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Acute myeloid leukaemia (AML), a cancer of the myeloid line of blood, is the most common myeloid leukaemia and is responsible for over 10,000 deaths each year. With chemotherapy being the only available treatment for AML patients to date, novel treatments are desperately needed. Smac mimetics are currently in clinical trials for the treatment of various cancers. These drugs antagonize inhibitor of apoptosis proteins (IAPs) and simultaneously induce TNF secretion to render cancer cells sensitive to TNF induced killing. We have previously shown that the combination of a Smac mimetic and p38 inhibitor achieves synergistic killing in AML (Lalaoui et al., Cancer Cell 2016). Furthermore, the inhibition of the MAPK p38 is able to overcome Smac mimetic resistances observed in some AMLs. The exact molecular mechanism by which p38 inhibitors increase Smac mimetic mediated killing is still unknown. Using a phosphoproteomic approach we therefore seek to identify mediators that are involved in the synergistic effect between Smac mimetics and p38 inhibitors. To achieve this, we have investigated time-dependent changes in the phosphoproteome of murine bone marrow-derived macrophages (BMDMs) treated with a Smac mimetic alone or in combination with a p38 inhibitor. We used a label-free quantitative phosphoproteomics approach whereby phosphopeptides were enriched using magnetic Fe-NTA beads. Subsequent analysis of our quantitative phosphoproteomics data revealed and enrichment of kinases with known roles in the observed synergism. Moreover, we have uncovered a number of phosphorylated proteins that potentially represent novel mediators of the mechanisms behind the synergistic killing of AML cells via Smac mimetics and p38 inhibitors. This research paves the way for new therapeutic targets in the treatment of AML.

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Proteomic and degradomic analysis of a virulence-associated serine peptidase from *Campylobacter jejuni*

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Campylobacter jejuni is one of the leading causes of acute gastroenteritis in the developed world, and a major antecedent for a number of debilitating autoimmune disorders. Recently, the serine peptidase Cj0511 has been identified as being required for optimum virulence and is associated with number of virulence mechanisms including biofilm formation, stress tolerance and pancreatic amylase triggered α-dextran secretion. As a component of outer membrane vesicles, which are a key mechanism for delivery of a number of classical mediators of pathogenicity including components into host cells, there is also an implication that Cj0511 may also modify host proteins during infection. Here, we employed iTRAQ-based labelling to determine the effect of loss of the peptidase on whole protein abundance in a hypermotile population of the original sequenced strain; *C. jejuni* strain 11168H. Of the 1306 *C. jejuni* proteins quantified, only 67 were deemed to have a significant change in abundance in the Δ*cj0511* strain

relative to the wild-type isolate. Of these, 41 were reverted to WT levels in a complemented strain, Δ*cj0511Ωcj0046* including a number of proteins from the *dccRS* regulon, flagellar components and protein secretory systems all of which have known associations with virulence. *N*-terminal amine isotopic labelling of substrates (*N*-TAILS) was also employed for a pair wise comparison of the *N*-degradome of wild-type 11168H and the *cj0511* knock out strain to both identify biological targets of the protease as well as attempt to elucidate the sequence specificity of the protease. These proteomics-based approaches were complemented with various standard phenotypic tests to further establish the role of the protease towards *C. jejuni*'s physiology.

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The mystery of tungsten: An unexpected bond with Cu,Zn-SOD

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Superoxide Dismutases(SOD) are a well-established family of enzymatic metalloproteins, functioning primarily to catalyse the disproportionation of superoxide that results from cellular respiration. Biophysical and biochemical characterisation of Cu,Zn SOD have shown that the protein's structure and enzymatic activity are dependent on proper metalation of the enzyme. SOD1 requires copper for catalytic function. Zinc is required to maintain the structure of the enzyme and promotes the formation of the dimeric form of the enzyme. Mis-metallation of SOD have been implicated as a primary cause of amyotrophic lateral sclerosis.

We noticed during the purification of SOD from bovine liver that SOD was found not only to contain copper and zinc, it had also incorporated tungsten. Tungsten(W), a non-essential trace element, is considerably understudied and often overlooked. Little has been uncovered regarding its biological relevance apart from limited literature detailing potential toxicity. The presence of W in the bovine liver SOD is likely due to trace environmental exposure. To further investigate the role of W incorporation in the toxicity of W we investigated W incorporation in SOD from mouse exposed to high levels of tungsten. We used metalloproteomics to understand the protein targets that incorporate tungsten during exposure. We characterised and tungsten-SOD1 complexes using liquid chromatography(LC) coupled to inductively coupled plasma mass spectrometry(ICP-MS).

As expected, tungsten treated mice were observed to have significantly increased levels of tungsten-containing proteins when compared to control mice. To further understand the consequence of tungsten incorporation into SOD an fi it is bound to the Cu or Zn site we produce apo-SOD and attempted to titrate tungsten into apo-SOD. This was unscuseessful under the conditions used and suggests that a molecular cofactor maybe necessary for tungsten incorporation. Our results have implications for acute tungsten toxicity and environmental exposure due to occupational exposure working with tungsten.

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Quantitative characterisation of the paediatric burn blister fluid proteome using SWATH MS to assist clinical diagnosis

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Burn injury is a common and traumatic event in the paediatric population. At present, the diagnosis of burn injury severity is largely dependent on the clinician's experience. A better understanding of the biochemistry of burn injury would assist with the development of objective and quantitative measures to aid diagnosis. Burn blister fluid (BF) is considered to be a viable source of biomolecules that reflect relevant systemic responses and the local microenvironment.

In order to generate a comprehensive peptide spectral library, a subset of BF samples were pooled according to burn depth (12 superficial (S), 12 deep-partial thickness (D), and 4 full thickness (F)) and fractionated by four different methods, including ultrafiltration, SDS-PAGE, OFFGel isoelectric focusing and immuno-depletion, prior to digestion and subsequent LC-MS/MS analysis in data-dependent acquisition mode. All individual BF samples (n=100) were then analysed using LC-MS/MS in data independent acquisition mode (SWATH) to obtain quantitative data.

More than 800 individual proteins were identified and formed the basis of a BF peptide spectral library. The relative ion abundance of more than 600 proteins in every individual sample was extracted and correlated with different clinical parameters, such as burn depth, time to re-epithelialisation, first aid treatment, and burn size. Analysis of these data using orthogonal partial least squares–discriminant analysis (OPLS-DA) revealed the key biochemical differences that stratify sub-groups within the clinically relevant parameters..

We have shown that the blister fluid proteome can be used to classify paediatric burn wounds by different burn depths and by other clinically relevant parameters. These markers are under further investigation to determine their viability as clinic

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The N-terminome of *Mycoplasma hyopneumoniae* reveals novel insights into protein processing

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During protein maturation, post-translational proteolytic processing events sculpt the proteome by cleaving single or secretion signals from the polypeptide chain, generating new proteoforms. *Mycoplasma hyopneumoniae* is a genome-reduced, porcine respiratory pathogen responsible for severe economic losses to swine production. We have characterised proteolytic events in key adhesin families, that occur at defined sites which are essential to the formation of functional, mature proteoforms on the extracellular surface of the organism. Some of these N-terminal processing events are performed by the ubiquitous methionine aminopeptidase (MAP) enzyme which removes the first methionine when the proceeding amino acids are small, in a manner that follows the N-end rule common to most organisms. To explore the extent of proteolytic processing we took an untargeted, high-throughput approach to identify neo-N-termini in the proteome of *M. hyopneumoniae*. Using protein dimethyl-labelling, reversed-charge enrichment of N-termini and mass spectrometry, 672 N-terminal sequences were characterised. Our data verified 74 protein start sites and identified the precise location of post-translational proteolytic cleavage events in 58 functionally-diverse proteins. Many of the protein N-termini identified adhere to the N-end rule, however we observed cleavage events which did not follow the N-end rule. We propose that other aminopeptidases, which are present in *M. hyopneumoniae,* are responsible for the removal of methionine in these proteins and contribute to the diversity of processing events in this important agricultural pathogen. We tested this hypothesis by assessing the ability of recombinant aminopeptidases to remove methionine from a panel of synthetic peptides with different amino acids in position two. Endoproteolytic cleavage sites were also identified in 35 proteins with well-defined, canonical functions in the cytosol. These observations suggest that microbial N-terminal processing of proteins is more complex and widespread than previously thought.

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A comprehensive proteomic study of cancer cell derived microparticles

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Microparticles, a form of extracellular vesicle, have been demonstrated to have a role in cellular cross-talk, affecting the cellular microenvironment in both normal and disease-states. The ability of cancer to exchange material between cells enables the dissemination of mechanisms for the tumours' survival and proliferation. Microparticles shed from tumour cells have been linked with malignancy, angiogenesis, immune system evasion, chemo-resistance or multidrug resistance, invasiveness of the cancer and coagulation abnormalities seen in many cancer patients. Thus, a thorough understanding of cell-to-cell communication through the action of microparticles is essential to understand cancer mechanisms.

Microparticles were isolated from a panel of cancer cell lines, originating from a variety of sites around the body and varying in aggression. Whole cell and whole microparticle lysates were analysed via LC-MS/MS and the protein profiles of each were examined. These show conserved proteins within the microparticles as well as many differences between the microparticle populations. These microparticles protein cargo were compared to their parental cells and some proteins are shown to increase in concentration within the vesicles. This alludes to proteins that may be important in microparticle formation and function or are selectively packaged for intracellular communication and transfer. This work aims to understand microparticles physiological role in cancer progression.

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Temporal profiling of protein phosphorylation during myocardial reperfusion injury

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Cardiovascular diseases are one of the leading causes of death worldwide with ischemic heart disease the largest contributor. To minimise ischemic damage, timely reperfusion is essential to salvage the affected tissue however, reperfusion itself can result in further damage. While a number of key signalling pathways are known to be involved in reperfusion injury, the key activators and temporal profile of such signalling proteins is unknown. The current study utilises large scale phosphoproteomics to monitor the activation/repression of signalling pathways during the reperfusion period. Rat hearts were subjected to 15 minutes of global ischemia (15I) by Langendorff perfusion with periods of reperfusion spanning 1-, 2-, 5-, 15- and 60-minutes. Peptides were labelled with TMT prior to enrichment of phosphopeptides utilising the TiSH method, with analysis by HCD tandem MS. This study identified 18,760 phosphopeptides originating from 4,915 proteins. Using a z-score cutoff of ±1 we observed 8,025 phosphopeptides significantly regulated over the timecourse of 60 minutes reperfusion, with 5,189 of these occurring within the first 2 minutes of reperfusion. These regulated phosphopeptides were mapped to functional pathways utilising STRING and KEGG to a number of signalling pathways including MAPK, cGMP-PKG and PI3K-Akt signalling as well as a number of pathways responsible for contractility and cellular structure including the actin cytoskeleton, calcium signalling, tight junction and focal adhesion. By elucidating potential signalling proteins responsible for the initiation of reperfusion injury, novel therapeutic targets are possible to improve cardiovascular outcome after an ischemic event, such as the focal adhesion kinase which shows >2 fold increase in phosphorylation indicative of increased activity and is a key activator of a number of signalling pathways which can result in apoptosis.

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In-depth mass spectrometry characterisation of *S. cerevisiae* protein methyltransferases: are they endpoints of signalling pathways?

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In recent years, it has emerged that methylation is a major intracellular post-translational modification (PTM) of proteins and modulator of protein-protein interactions (PPIs). In yeast, over twenty protein methyltransferases (MTases) have been identified. Their substrates include RNA processing proteins, the translation machinery and histone proteins, on which dozens of lysine and arginine methylation sites have been mapped. The regulation of protein MTases, however, is poorly understood. To investigate whether protein MTases could be subject to post-translational regulation, we overexpressed and purified the following *S. cerevisiae* protein MTases from their native host: Hmt1, Set5, Rkm1, Rkm4 and Efm4. This set of MTases includes both lysine and arginine methyltransferases, and encompasses the different types of MTases present in yeast in regards to structure and function. Then, we fully characterised these MTases using a multi-protease, multi-fragmentation mass spectrometry workflow. Briefly, the purified MTases were digested with trypsin, LysargiNase or Asp-N, then each sample was analysed by LC-HCD-MS/MS and LC-ETD-MS/MS. With this approach, we identified several novel phosphorylation, acetylation and methylation sites on these enzymes. By projecting each PTM to structural models of the MTases, potential regulatory PTM sites and PTM hotspots were revealed. In particular, some PTMs fell on the same interface as the catalytic pockets of Rkm1 and Efm4, suggesting a potential role for these PTMs in mediating the enzyme-substrate interaction. Other PTMs fell in predicted disordered regions of Hmt1, Set5 and Rkm4, which is a common feature of regulatory PTMs. Altogether, these results reveal that protein MTases are highly modified enzymes and suggest several of these PTMs have regulatory functions, opening the venue to further research on the regulation of protein methylation.

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METTL21B is a novel human lysine methyltransferase of translation elongation factor 1A: discovery by CRISPR/Cas9 knock out

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Lysine methylation is widespread on human proteins, however the enzymes that catalyse its addition remain largely unknown. This limits our capacity to study the function and regulation of this modification. Here we report that human METTL21B is a protein methyltransferase, which methylates lysine 165 of eukaryotic translation elongation factor 1A (eEF1A). The CRISPR/Cas9 system was used to knock out putative protein methyltransferases METTL21B and METTL23 in K562 cells. The known eEF1A methyltransferase EEF1AKMT1 was also knocked out as a control. Targeted mass spectrometry revealed the loss of lysine 165 methylation upon knock out of METTL21B, and the expected loss of lysine 79 methylation on knock out of EEF1AKMT1. No loss of eEF1A methylation was seen in the METTL23 knock out. Recombinant METTL21B was then shown to catalyse methylation on lysine 165 in eEF1A1 and eEF1A2 in vitro, confirming it as the methyltransferase responsible for this methylation site. METTL21B is specific to vertebrates, with its target lysine showing similar evolutionary conservation. We suggest METTL21B be renamed eEF1A-KMT3. This is the first study to specifically generate CRISPR/Cas9 knock outs of the genes encoding putative protein methyltransferases, for the purpose of substrate discovery and site mapping. Our approach should prove useful for the discovery of further novel methyltransferases, and more generally for the discovery of sites for other protein-modifying enzymes.

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Development and validation of a pipeline for the biophysical characterization therapeutic monoclonal antibodies using MALDI-HXMS

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Hydrogen deuterium exchange followed by Mass Spectrometry (HXMS) is a biophysical tool capable of probing protein/ligand interactions, conformational changes, and protein dynamics. Despite an increased number of applications, the expansion of the technology has been slowed by its intrinsic technical and analytical complexity (*i.e.*, sequential digestion at pH 2.5 and rapid HPLC separation at 0°C). Although many HXMS studies have been conducted with electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) mass spectrometry turns out to be a convenient tool for this purpose. MALDI-HXMS combines the advantages of high speed of analysis and excellent sensitivity and accuracy of mass measurements with the capability to analyse peptide maps in a single spectral acquisition. With recent advancements in sample preparation robotics entire MALDI-HXMS experiments (typically 64-128 samples) can be executed in less than one hour.

We have developed an integrated R-package named MALDI-HDX to help analyse large MALDI-HXMS datasets. MALDI-HDX uses the MALDIquant Foreign R package to import Bruker MALDI-TOF data. A viewer facilitates semi-automated assessment of the quality of all spectral segments across an XIC peak for centroid mass determination. The resulting HXMS data are then subjected to statistical analysis using the MEM-HDX R package. This interactive tool allows the user to validate, visualize and

compare the relative deuterium incorporation on the amino acid sequence and 3D structure, providing both spatial and temporal information.

The biophysical characterization of therapeutic monoclonal antibodies recognizing different epitopes establishes the ability of MALDI-HXMS to provide useful information. This approach may be extended to supramolecular interactions, provided that peptide mapping is conducted carefully in order to deliver unambiguous sequence coverage. MALDI-MS alone might not be accurate enough to achieve successfully this task in all cases, and should then be associated to LC-MS/MS sequence assignment for the first part of HXMS methodology.

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Characterising T helper cell epitopes from the envelope protein of HIV

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Virus-specific CD4+ T-cells are essential for maintaining an effective immune response by providing "help" to B-cells and CD8+ T-cells. They may also have a direct cytolytic activity against infected cells. The T cell receptor (TCR) on the surface of human CD4+ T-cells recognises antigenic peptides presented by the class II HLA molecules, HLA-DR, -DQ or -DP. Recognition of these peptide-MHC complexes by CD4+ helper T-cells facilitates B-cell activation, proliferation and the secretion of virus-specific antibody. Most of the HIV epitopes recognised by CD4+ T-cells have been identified by measuring T-cell responses against overlapping peptide libraries. In order to identify the naturally processed and presented forms of HIV envelope derived peptide antigens, HLA class II molecules were immunoaffinity purified from the surface of HIV+ antigen presenting cells and their peptide cargo sequenced by high-resolution mass spectrometry. Using this approach, seventeen epitopes were identified from HIV antigens, of which nine are novel. Four of these novel epitopes span the ectodomain near the N-terminus and are part of the Heptad repeat region 1 which plays a crucial part in viral entry into host cell. A nested set of five epitopes are located on the cytoplasmic tail of envelope and covers a site that is involved in NFKB activation. The epitopes identified will help widen the search for T helper determinants that drive antibody production *in vivo*. In addition to the HIV peptides, more than 30000 endogenous HLA class II ligands were identified. This represents the largest data set of class II bound peptides generated to date and has provided new insights into the origin and properties of naturally presented HLA class II ligands. This large dataset of HLA-bound peptides will also enable development of more accurate prediction algorithms that will aid in development and identification of CD4+ determinants in HIV and other diseases.

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Identification of the dominant endogenous factors regulating inflammation and regeneration in skeletal muscle following physical trauma

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Muscle injury is a prevalent cause of debilitation for workers, athletes, and the public generally at home and in motor vehicle accidents. These incidents cause suffering to patients and even permanent disability. To date, a number of physical trauma models have been established, including *in vivo* which lay a foundation of research into skeletal muscle injury. While the general processes associated with skeletal muscle injury have been described.

In spite of these investigations into skeletal muscle injury, it is still unclear as to the specific mechanisms of injury progression and factors affecting the initiation of the recovery process. It is therefore necessary to conduct research aimed at providing more detailed insights into the fundamental factors and mechanisms that regulate the activity of skeletal muscle cells following injury. This may further enable the development of potential novel treatment approaches for improving the recovery from serious muscle trauma.

Methodology

The project will combine global/profiling and functional proteomics based approaches in *in vivo* model. Specifically, a rat impact contusion model will be utilised to model impact trauma. Dynamic global protein profiling using, LC-MS/MS will be performed at 6h, 12h, 1, 3, 7 and 14 days on tissue homogenates to identify factors that are associated with the initial recovery response following injury.

Expected Results

1. There are temporal changes to biochemical pathways and processes during recovery from muscle trauma *in vivo*, and global proteomics profiling will reveal these changes.

1. Dominant endogenous factors present in injured muscle will activate signalling pathways resulting in secretion of cytokines that regulate inflammation and regeneration in skeletal muscle.

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MALDI Mass Spectrometry Imaging Reveals Different Proteomics Patterns between Vulvar Cancer With and Without Lymph Node Metastasis

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Vulvar cancer is the fourth most common gynecological cancer worldwide. However, limited studies have been completed on the molecular characterization of vulvar squamous cell carcinoma resulting in a poor understanding of the disease initiation and progression. Analysis and early detection of the precursor lesion of HPV-independent vulvar squamous cell carcinoma (VSCC), differentiated vulvar intraepithelial neoplasia (dVIN), is of great importance given dVIN lesions have a high level of malignant potential. Here we present an examination of adjacent normal vulvar epithelium, dVIN, and VSCC from vulvar cancer patients by peptide Matrix-assisted laser desorption/ionization Mass Spectrometry Imaging (MALDI-MSI). The results reveal the differential expression of multiple peptides from the protein cytokeratin 5 (CK5) and other cytoskeletal proteins across the three vulvar tissue types. The difference observed in the relative abundance of CK5 by MALDI-MSI between the healthy epithelium, dVIN, and VSCC was further analyzed by immunohistochemistry (IHC) in tissue from eight VSCC patients. A decrease in CK5 immunostaining was observed in the VSCC compared to the healthy epithelium and dVIN. These results provide an insight into the molecular fingerprint of the vulvar intraepithelial neoplasia that appears to be more closely related to the healthy epithelium than the VSCC.

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Multi-omics approach to understand how *Pseudomonas aeruginosa* adapts to the cystic fibrosis lung

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Pseudomonas aeruginosa is an opportunistic bacterial pathogen commonly associated with chronic lung infection in cystic fibrosis (CF). To better understand the molecular mechanisms underpinning adaptation of the bacteria to the CF lung microenvironment we conducted genomic, proteomic and phenotypic characterisation of four novel CF isolates of *P. aeruginosa* cultured in various growth media (Synthetic Cystic Fibrosis Media, Luria-Bertani (LB) broth, M9-Glucose) and under oxygen stress (O_2<1%). Genomic and phenotypic analyses revealed significant diversity in colonization, virulence and metabolic traits different to a reference laboratory strain PAO1. Whole cell and membrane proteome profiling using combinations of iTRAQ, TMT and SWATH mass spectrometry enabled quantification of 3,849 proteins from whole cell extracts and 990 membrane proteins from membrane enriched fractions (FDR <1%) mapping around 71% of predicted ORFs and representing the most comprehensive proteome of clinically relevant *P. aeruginosa* reported to date. CF clinical isolates shared a core proteomic signature which is different to PAO1. In comparison to PAO1, we observed down-regulation of motility, adhesion and chemotaxis proteins (FliK, PilJ, PctA), questioning the utility of targeting these molecules for vaccine development. We also saw up-regulation of proteins used in respiration (*Cbb_3-1*,*Cbb_3-2*,NarG-I, NirC, NorB) and drug resistance (MexY, MexB, MexC). Functional assays including antibiotic MIC assay, motility and CF sputum adhesion assays confirmed the proteomic findings. These proteome maps and phenotypic profiles illuminate the diversity in the adaptation and micro-evolution mechanisms of *P. aeruginosa* that cannot be detected at the genome level alone.

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Using an integrative multi-omics approach for the identification of novel quantitative biomarkers for artemisinin resistance in *Plasmodium falciparum* parasites

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The emergence of artemisinin resistance in the malaria parasite, *Plasmodium falciparum* poses a major threat to the control and elimination of malaria. The underlying mechanism associated with artemisinin resistance is poorly understood, but point mutations in the PfKelch13 protein strongly correlate with resistance. However, using *PfKelch13* genomic sequence as a molecular marker is resource-intensive and has limited sensitivity and specificity, as mutations do not always lead to resistance to artemisinin. Therefore this study aimed to identify novel quantitative biomarkers for artemisinin resistance using an integrative multi-omics approach, which combines proteomics, peptidomics and metabolomics to analyse global differences between drug-resistant and drug-sensitive parasite strains.

When applied to *P. falciparum* infected red blood cells, our multi-omics platform facilitated the identification of approximately ~2300 proteins, 800 putative metabolites and 971 naturally abundant peptides. Proteomics analysis from three independent artemisinin resistant lines found artemisinin resistance to be associated with decreased expression of PfKelch13 protein by approximately 2 fold. As the *PfKelch13* sequence is already a genetic biomarker for artemisinin resistance, we show for the first time that the abundance of this protein is also associated with the resistance phenotype.

Metabolomics analysis from two independent artemisinin resistant lines showed significant accumulation of glutathione and a reduction in the abundance of nine metabolites, primarily involved in amino acid metabolism. Peptidomics analysis revealed lower

abundance of several endogenous peptides derived from haemoglobin (HBα and HBβ) in the resistant strains. This dysregulation of specific endogenous metabolites, peptides and the PfKelch13 protein itself, provides additional insight into the mechanism of artemisinin resistance, and novel candidates which could potentially be used as quantitative biomarkers for artemisinin resistance.

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The Glaucomic eye: A window into the molecular basis of Alzheimer's disease

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The association of glaucoma with Alzheimer's disease (AD) has been postulated by several recent studies. Both diseases are neurodegenerative, chronic, and present as a continuum. Also, diagnosis is poor in the early, rather than later, stages of the disease process. We have previously demonstrated that AD is associated with ocular deficits including retinal thinning and reduced electrophysiological response. However, the molecular basis of this link remains obscure. This study was designed to elucidate the association between glaucoma and AD by investigating glaucoma-associated protein changes in the retina and vitreous humour. We performed multiplexed proteomics (TMT labelling) using the SPS-MS3 method on an Orbitrap Fusion mass spectrometer on retinal tissue and vitreous humour fluid collected from 12 glaucoma patients and 12 age-matched healthy controls. Detailed functional and protein-protein interaction analyses were performed using complementary pathway analysis tools. We observed the differential regulation of 51 and 105 proteins, in retinal and vitreous tissues, respectively, that are in functional networks associated with AD pathology. Moreover, the differential expression of selected "research only" AD biomarkers were further evaluated using Mesoscale Discovery 96-Well MULTI-SPOT assay platform and western blotting. Taken together, our findings are in strong agreement with the inverse Warburg Hypothesis, which suggests that glaucoma and AD occur in response to mitochondrial dysfunction and metabolic reprogramming. Moreover, the classical complement pathway appears to be activated in both glaucoma and AD, suggesting a common innate inflammatory response in both disorders. Our data provides the first biochemical evidence that may explain the pathological similarities and differences between glaucoma and AD. It also provides renewed understanding of the roles of neuro-energetics in the aetiology and pathogenesis of age related neurodegenerative diseases.

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Predicting Motif Mimicry in Viruses using Protein-Protein Interaction Data

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Many viruses hijack host cellular machinery through mimicry of Short Linear Motifs (SLiMs) that interact with host protein domains. SLiMs are short stretches of amino acids (~3-10) which are involved in post translational modifications (PTMs), protein-protein Interactions (PPIs), cell regulation and cell compartment targeting. To date, several studies have been conducted to identify PPIs, but no specific study to see how well different PPI-capturing methods capture SLiMs-mediated interactions. The main objectives of this study are: 1) to predict Domain Motif Interactions (DMIs) among viral and host proteins; 2) to find whether virus-human PPI data from the virhostome resource is enriched for DMIs; and 3) to see which PPI method is better for studying DMIs. Results have shown that virhostome data is enriched for DMIs and can be a good source to study motif mimicry in viruses. Permutation tests showed more enrichment for DMI in TAP data than Y2H data. Moreover, novel candidate DMIs have been discovered which need further validations. The outcome of this study will be helpful in uncovering unique strategies of viruses to interact with human proteins which will eventually be significant for pathogen research.

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Comparative analysis of acute and chronic phenotypes of the *Pseudomonas aeruginosa* Australian Epidemic Strain AES-1 in a Cystic Fibrosis lung infection model

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Pseudomonas aeruginosa is the most widely distributed infecting organism within the Cystic Fibrosis (CF) community. It accounts for a significant degree of morbidity and mortality, and is associated with vastly shortened lifespan. In the dehydrated mucus of CF lungs, CF-associated *P. aeruginosa* form antibiotic resistant biofilms leading to chronic infection. Little in form of effective treatments exist, and vaccine candidates to date provide little to no protection. Sequentially isolated isogens of clinical *P.*

aeruginosa representing an acute infecting phenotype (AES-1R), and 10 years of within-host adaptation (chronic infecting phenotype: AES-1M) were grown in a medium mimicking CF sputum to characterise process of adaptation and subsequently identify proteins associated with initial colonization of CF lungs. Proteins harvested from lysates at 18h (early infection) and compared to 48h (early biofilm), 72h (mature biofilm) and 96h (dispersing biofilm) were trypsin digested and iTRAQ labelled to quantify differences during AES-1R and AES-1M adaptation to CF lung. Additional experiments compared between-phenotype differences (AES-1R versus 1M) at 18h and 72h. Approximately 70% of total proteome was identified, with differences in abundance over time including pathways associated with early establishment of infection (e.g. type III/VI secretion pathway and motility). Adaptation trends over time were comparable with 2151 proteins commonly expressed between AES-1R and AES-1M over all four time points; whilst 575 proteins were differentially abundant between two phenotypes at 18h and 1059 proteins at 72h (FDR <0.01). AES-1R/1M differences included pyocyanin and pyoverdine biosynthesis. AES-1R displays a more virulent phenotype, with characteristics associated with increased capacity for biofilm formation, whilst many pathways associated with persistence are upregulated in AES-1M. Results obtained are a detailed analysis of adaptation of *P. aeruginosa* to CF sputum, and resulted in identification of 6 commonly expressed proteins important in persistence for use in further *in vivo* immunogenicity trials as possible vaccine candidates.

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Unravelling the proteomic profile of rice mitosis under drought stress

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Stem cells play a critical role in the regulation of growth and development of multicellular species. In rice, the shoot growing zone comprises a small population of stem cells, self-renewing cells which can differentiate into diverse specialized organs and tissues such as stems, leaves and flowers. Drought effects on rice have been studied extensively, however, the effect of water deficit on the development of rice growing zone cells which are protected by the surrounding mature leaf tissue, has not been explored. Two rice cultivars with contrasting genetic backgrounds and levels of tolerance to drought, Nipponbare and IAC1131, were used in this study. Four-week-old seedlings of both cultivars were grown in large soil volumes and then exposed to moderate and extreme drought for 7 days, followed by 3 days of re-watering. Shoot growing zone tissues were harvested from plants from each treatment for protein extraction and subsequent shotgun proteomic analysis. Gene Ontology (GO) annotations of differentially expressed proteins provide insights into different developmental processes of Nipponbare and IAC1131. Our data indicate that IAC1131 appears to be better able to cope with stressful conditions by up regulating a suite of proteins potentially involved in cell division, developmental process, cellular component organization and cell growth, to maintain the active state of the growing zone. Nipponbare, on the contrary, lacks the range of stress responses shown by the drought tolerant variety, and the cell division activity is severely impaired by drought stress.

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Proteomic study of beta-catenin protein interactions in colon cancer

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β-catenin is a protein well-known for its association with several human cancers and is known to accumulate in the nucleus with cancer progression. It is a multi-functional protein that can be found at different subcellular localisations, regulated in part by protein interactions and phosphorylation.

Here, β-catenin protein interactions in colon cancer were investigated and explore the role of phosphorylation at amino acid Y654 in regulating these interactions was explored. Recombinant human full-length β-catenin constructs were used as affinity baits to isolate protein binding partners from stable isotope labelled SW480 and HT29 colon cancer cell lines while high-resolution mass spectrometry (Orbitrap ELITE) was used for protein identification and quantitation.

For wildtype β-catenin (Y654), 379 and 123 putative protein interactors were detected in SW480 and HT29 respectively. For the phosphomimetic (Y654E) construct, 92 and 224 putative protein interactors were detected in SW480 and HT29 respectively. For the non-phosphorylatable (Y654F) construct, 129 and 48 putative proteins interactors were detected in SW480 and HT29 respectively.

A number of these candidate binding proteins were further validated by immunoprecipitation and immunofluorescence and strongest evidence was shown for mitochondrial HSP70 – providing both a novel interaction and localisation for β-catenin.

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Quantitative Proteomics to Explore the Thiol Proteome of Duchenne Muscular Dystrophy

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Oxidative stress, caused by excessive reactive oxygen species (ROS), is evident in many chronic diseases. Protein thiol oxidation is the focus of this research because thiol groups (-SH) on cysteine residues of proteins are particularly sensitive to oxidation. As a consequence, changes in protein function have the potential to cause cellular dysfunction. In preliminary work using gel

electrophoresis and mass spectrometry identification, fourteen proteins were observed to undergo thiol oxidation in muscles of a dog model for dystrophy. Ten of these proteins were identified and found to be located in various subcellular compartments including the cytosol, nucleus, mitochondria and also in the extracellular space. Of particular note, lactotransferrin showed a remarkable increase in thiol oxidation of dystrophic muscle. Lactotransferrin is found in the granules of neutrophils, which suggests that neutrophils could be a major source of oxidative stress in the pathogenesis of dystrophy.

These data show that cell and tissue dysfunction could be a collective response to the thiol oxidation of multiple proteins. Expanding the number of proteins that can be identified as being oxidised would improve understanding of how protein thiol oxidation is causing pathogenesis in dystrophy. We propose proteomic methods (high throughput proteomic techniques using gel-free mass spectrometry) to elucidate protein location, involvement in molecular networks and to elucidate molecular pathways that contribute to cellular dysfunction in muscular dystrophy.

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Exploring the consequences of genetic variation and associated proteomes in chronic venous leg ulcers

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Venous leg ulcers (VLU) are debilitating wounds that can remain unhealed for several decades and recur in up to 70% of cases. The progression and recalcitrance of the condition is not well understood and, although there is evidence to suggest a genetic predisposition, the genes involved have yet to be elucidated.

A suite of six candidate genes has been selected based on their proposed involvement with venous health. Using a genotyping approach these genes will be analyzed in VLU patients and age matched controls while the protein in patient wound fluid samples will be analyzed using quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS). The abundance of proteins associated with the target genes and related biochemical pathways will be a focus of this study, in addition to comparison of wound fluid biochemistry to VLU associated clinical parameters. Bioinformatic integration of genetic and proteomic datasets will be performed to determine if there is a genetic link to the biochemistry that underpins non-healing venous leg ulcers. This will be performed using gene ontology and pathway enrichment in applications such as Reactome, Ingenuity Pathway Analysis and Cytoscape. We hypothesise that the genomic analysis will demonstrate a differential expression of wildtype and variant genes between the control and patient cohorts for some or all of the six target genes. Moreover, it is expected that quantitative proteomics of ulcer fluid will show differential abundance in the protein profile between healing ulcers and recalcitrant wounds. In addition, it is anticipated that proteins associated with the target genes will have an altered abundance in non-healing or slow healing wounds. Finally, through integration of our datasets, we expect to find that patients with variant copies of genes will display clinical symptoms that correlate with the physiological role or pathway of the gene as measured at the protein level.

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Characterising the role of protein kinase CK2 in regulating aluminium toxicity in yeast

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Aluminium (Al^{3+}) is a non-essential metal ion and chronic exposure is considered toxic. Chronic Al^{3+} accumulation has been linked to cancers, neurodegenerative disorders such as Alzheimer's disease, and is a major constraint to crop yield in acidic soil. Despite its insidious risks, aluminium compounds are constantly used in anti-perspirants, adjuvants, foods and beverages. Therefore, the fundamental insights into the mechanism of Al^{3+} toxicity need to be investigated. Through a genome-wide screen of the deletion mutants of *Saccharomyces cerevisiae*, we found protein kinase CK2 to be a key player in regulating Al^{3+} toxicity. CK2 is a highly conserved serine/threonine protein kinase consisting of two catalytic subunits and two regulatory subunits. Deletion of the catalytic subunit *CKA2* provides resistance to Al^{3+}. Five strains of *S. cerevisiae* (Al^{3+} sensitive BY4743, *cka1*Δ, *ckb1*Δ and *ckb2*Δ and Al^{3+} tolerant *cka2*Δ) were treated with 1.6mM Al^{3+} and H_2O for the control in a growth time-course (0 to 16 hr) and performed in duplicate. Reverse phase chromatography was used for the creation of the spectral library, generated with ProteinPilot™ 5.0 and searched against the yeast Uniprot 2016 database, in which 3196 proteins (1% FDR) were identified. The generated data was imported into PeakView™ 2.1 and matched against SWATH-MS data in which 2283 proteins were quantified. Statistical analysis of the differentially expressed proteins was performed using Perseus 1.5.5.3. In the Al^{3+} sensitive strains, Al^{3+} damages the cell wall, plasma membrane and disrupts the anti-oxidant response, which explains the sensitive phenotype of these strains. For Al^{3+} detoxification, the key findings of the protective role of *cka2*Δ against Al^{3+} include overexpression of proteins in sulfur metabolism, lysine biosynthesis and the heat shock response. These novel findings provide in-depth understanding of Al^{3+} toxicity and its detoxification, and have implications in finding solutions to Al^{3+}-related problems in health and agriculture.

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Effect of Serum Concentration on the Proteome of Rat Bone Marrow-Derived Mesenchymal Stem Cells

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Mesenchymal Stem Cells (MSCs) could be a potential treatment for multiple diseases and injuries due to their ability to replenish many cell types in the human body. Translation of this requires cells to be maintained in their non-differentiated state, which remains a major challenge in the field. Optimal conditions for culturing these cells are now required; thus, in this study, rat MSCs cultured with three different supplemental serum concentrations were investigated using a proteomics approach. Bone marrow-derived rat MSCs were established in medium containing 10% serum. Cells were then starved prior to the application of media with different concentrations of serum (0%, 2% or 10%) and cultured for 24 hours. Cellular protein was collected and prepared for qualitative and quantitative (SWATH) mass spectrometry using standard techniques. Multivariate analysis (PCA, PLS-DA and oPLS-DA) revealed biochemical differences across treatments. Gene ontology (GO) enrichment analysis was used to determine the biological processes of cells in response to treatments. A protein library containing 803 proteins was generated, 58.03% of which were observed in all three treatments, while 8.47%, 8.47% and 1.49% were unique to 0%, 2% and 10% serum, respectively. Fewer proteins were detected in the 10% serum group compared to lower concentrations possibly due to high abundant protein induced ion suppression. Based on the biochemical data, there was a more pronounced similarity between the serum free and 2% treatments compared to the 10% treatment. However, it is clear that unique biochemical features exists within all three treatments and these features may provide the insight needed to monitor and maintain MSCs in long term culture.

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SWATH-MS proteome profiling of copper-stressed *Pseudomonas aeruginosa* PAO1 provides leads for functional characterization

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The opportunistic pathogen *Pseudomonas aeruginosa* is well known for its environmental and metabolic versatility, and large genome of 6.3Mbp. Many proteins are yet to be functionally characterized and the expression of some remain to be verified. In this study, a proteome profile was produced of copper (Cu^{2+}) stressed *P. aeruginosa* PAO1 membrane and whole cell proteome fractions through the data-independent analysis technique SWATH-MS, resulting in ~2000 non-redundant proteins being quantified. Of these proteins 83 were found to be differentially regulated when cultured under stress from excess copper (±1.5, p-value <0.01). Examples of new proteins shown to respond to copper stress include: PA2807, a protein predicted to have a role in copper binding; proteins PA2064 and PA2065, proteins that have strong sequence similarity to the copper-related CopA and CopB proteins of *Pseudomonas syingae*; PA2505, a protein previously identified for the specific uptake of tyrosine, but now hinted to be involved in the non-selective diffusion of ions across the membrane, and finally, PA3920, currently described as a probable metal P-type ATPase, but now shows evidence of being a specific active transporter of Cu^{2+}, due to strong up-regulation observed in this study and accompanying biological/molecular function predictions. Additionally, it has been predicted that approximately 361 proteins of PAO1 have now been experimentally verified as a result of this study. This equates to 6.3% of the predicted PAO1 proteome where we now have experimental evidence for the expression of these proteins.

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Proteomics of Caco-2 human intestinal epithelial cell response to *Campylobacter jejuni* infection

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Campylobacter jejuni is a Gram-negative microaerophilic bacterium that is the leading cause of food-borne gastroenteritis in developed countries. The common route of human infection is *via* consumption of poorly prepared or under-cooked poultry, in which *C. jejuni* is an asymptomatic commensal. Human disease is characterised by mild to severe inflammatory diarrhoea, vomiting and inflammation. *C. jejuni* has also been associated with post-infection immune-mediated complications such as Guillain-Barre Syndrome, reactive arthritis and irritable bowel syndrome. The molecular basis for *C. jejuni* infection includes initial adherence to, followed by invasion of, human intestinal epithelium; however there remains limited knowledge on both the molecular basis of pathogenesis and the host cell response. Human intestinal Caco-2 cells were co-cultured with the pathogenic chicken colonizing isolate *C. jejuni* NCTC11168 O during a time-course of infection. Multiplicity of infection (the ratio of bacteria to mammalian cells) was determined to elucidate the characteristics of *C. jejuni* adhesion to, and invasion of, intestinal cells. The infection efficiency (total vs invading), intracellular survival (viability after prolonged co-incubation) and the cytotoxicity of *C. jejuni* in Caco-2 cells were determined. A temporal profile of Caco-2 cell response to *C. jejuni* infection was examined by quantitative proteomics, glycoproteomics and glycome profiling using liquid chromatography and tandem mass spectrometry. The results and methodologies that were used to characterise *C. jejuni*-mediated exploitation of host cell biology will be presented and discussed.

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Analysis of proteoforms in membrane protein complexes by top-down proteomics

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Membrane protein complexes are involved in essential cellular processes such as cell-cell communication, cell adhesion, transport, and energy homeostasis. Our current understanding of their function in health and disease is often limited despite their importance. Insight into the chemical structure of membrane complexes is fundamental to understand their biogenesis, function, and regulation. Analysis of the diverse proteoforms within a complex can be achieved by studying purified complexes via intact protein tandem mass spectrometry or "top-down proteomics". The hydrophobic or fragile nature of these complexes makes the task more difficult. We have developed a highly efficient method for the reproducible and unbiased extraction of intact subunits from excised clear native gel bands for top-down proteomics.

This top-down proteomics approach was validated by studying the five protein complexes of the mitochondrial oxidative phosphorylation (OXPHOS) system in *Bos taurus*. The OXPHOS enzymes are hydrophobic heteromeric complexes in the range of 0.2 – 1 megadalton that generate adenosine triphosphate to fuel cellular processes. A mitochondrial fraction was prepared from bovine heart,extracted protein complexes were subjected to high resolution clear native gel electrophoresis, and gel slices were cut from the gel to isolate OXPHOS complexes. The reproducibility and recovery of the extraction method was evaluated by denaturing gel electrophoresis and top-down proteomics using liquid chromatography with online ultra high resolution quadrupole time-of-flight tandem mass spectrometry. Denaturing gel electrophoresis results showed that all subunits from each complex were extracted without any bias. The vast majority of subunits for each complex could be detected by top-down proteomics except for some large or hydrophobic subunits. Various post-translational modifications were found such as mitochondrial import sequence cleavage, acetylation, formylation, and phosphorylation. In-depth characterization of proteoforms by electron transfer dissociation and collision induced dissociation tandem mass spectrometry will be presented.

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A Repository of Proteomics Software Tools

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The Proteomics group at the Walter & Eliza Hall Institute of Medical Research (WEHI) continue to develop and provide free software to the Australian Proteomics community. The software tools and packages are available from http://www.wehi.edu.au/people/andrew-webb/1295/andrew-webb-resources. Originally software tools were aimed principally at Mascot users due to the popularity of the Mascot search engine accessed through the Australian Proteomics Computationally Facility (APCF). This resource is still provided freely to academic researchers in Australia even though it is no longer grant funded. Additional software and resources such as "Download tools", "Sequence Databases" as well as "contaminants" are updated periodically. More recently software was developed for converting "MaxQuant" peaklists (APL) to generic peaklists (MGF) for usage in current and legacy software (e.g. denovo sequencing). A newly developed multi-threaded "MascotParser" is available which provides an easily configurable output format that is not available in the current download options. The latest most exciting software tool is MSCypher which brings together multiple freely available software programs such as alternative search engines (Digger, Comet, MSGFplus, Andromeda etc.) plus retention time prediction (Elude), machine learning tools (Random Forest) and the ability to automatically run R scripts such as Limma/Voom and/or MSstats. The MSCypher workflow is entirely user configured and command line. A graphical user interface, cloud-based access, open-mass searches are under development. Support for the PEFF sequence database format will also become available to better support variants, mutations and SNPs.

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Extracting more from large assay libraries using Gas Phase Fractionation SWATH-MS

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SWATH-MS advanced from using 32x25 m/z windows to cover the 400-1200 m/z MS1 range, to utilize variable windows (vW), with widths dependent upon peptide precursor density in given MS1 m/z ranges, leading to deeper proteome coverage, especially in the tryptic peptide rich region of 600-800 m/z. For plasma biomarker studies, despite deploying vW-SWATH, information extraction commonly only penetrates a small proportion of large plasma protein assay libraries available in community repositories. Therefore, we explored gas phase fractionation (GPF) SWATH to narrow MS1 windows and determine the impact on quantitative proteome profiling.

Tryptic digests of human plasma and SW480 cell lysates were analysed by 60 min GPF-SWATH and vW-SWATH on 6600 triple TOF (SCIEX). 400–600 m/z and 600–800 m/z with 4Da fixed windows, and 800-1200 m/z with 8Da windows were chosen for GPF-SWATH. The vW-SWATH method used 100 windows from 400–1200 m/z. A high pH fractionation assay library was used for SW480 cells and an online repository assay library for plasma.

GPF-SWATH was optimized with SW480 lysates. Information extraction from vW-SWATH using the high pH assay library revealed quantitative information for 2,600 proteins (9,800 peptides). Analysis using GPF-SWATH resulted in ~3,500 proteins (30% increase) and ~15,500 peptides (60% increase). Striking improvements were seen for 400–600 m/z with >2-fold increased peptide extraction and 1.5-fold increases for 600–800 m/z and 800–1200 m/z regions. Using an online repository assay library vW-SWATH extracted quantitative information for 300 proteins (1,500 peptides) from plasma. With GPF-SWATH, proteins with

quantitative information increased by 25%, peptides increased by 20%. Proteins unique to GPF-SWATH are less abundant cellular proteins, shed membrane proteins and extracellular matrix proteins.

GPF-SWATH of SW480 cells and human plasma demonstrates quantitation improvements over vW-SWATH and is a useful approach to take advantage of large assay libraries for deeper proteome quantitation.

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Optimisation of a tissue lysis and digestion protocol using pressure cycling technology (PCT)

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The recent combination of pressure cycling technology (PCT) and sequential window acquisition of all theoretical fragment ion spectra (SWATH)-MS, allows complex proteomic mass spectrometry data to be generated from small tissue biopsies (1-2mg) in less than 12 hours. ProCan aims to analyse 70,000 different cancer tissues over 7 years, utilising PCT-SWATH methodology. Optimisation of sample preparation steps is crucial for the success of this large scale, industrial analysis. The conventional sample preparation method utilises urea for protein denaturing and solubilisation. Urea is a well-used lysis buffer in shotgun proteomics but has a number of drawbacks. Concentrations of >1M have a detrimental effect on trypsin activity; it can introduce unwanted carbamylation of N-termini and lysine residues, when used at elevated temperatures, along with traditional 14-16 hour incubations; and it is used in high 6-8M concentrations, which requires desalting prior to MS injection. Sodium dodecylsulfate (SDS) is a detergent well known for protein solubilisation, but difficult to remove downstream, incompatible with LC-MS/MS analysis, and high concentrations are also known to effect trypsin activity. Sodium deoxycholate (SDC), on the other hand, can be easily removed with a simple acid precipitation step, and can be used at higher concentrations without affecting trypsin digestion. Using rat kidney biopsy punches the PCT protocol was optimised using various buffer components, and conditions. A combination of SDC and N-propanol instead of urea resulted in comparable numbers of protein IDs to that of the standard urea protocol. It produced a shorter, more efficient sample preparation method and resulted in the elimination of sample clean-up via solid phase extraction (SPE) prior to MS injection, thus improving sample throughput.

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Lipidomics Quantitation Using a Novel Scanning Quadrupole DIA Method

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Introduction

Targeted LC-MS assays are increasingly applied in the post-discovery omics area with an emphasis on validation. A novel DIA mode of operation, which provides both qualitative and quantitative information, has been developed for a tandem quadrupole/oa-time-of-flight (ToF) MS and applied to targeted lipidomics experiments using transition extraction lists and compound library based approaches.

Method

LC separation was performed using a Waters Acquity UPLC equipped with a 2.1mm x 100mm column. The *m/z* isolation range of the quadrupole was continuously scanned with MS data acquired up to a frequency of 2000 spectra/s. Alternate MS scans comprised precursor and CID product ions. The resulting 2D data, *m/z* (ToF) *vs. m/z* (quadrupole) were processed using Progenesis and Skyline informatics.

Results

Quantitative proof-of-principle data were acquired by serially diluting lipid standards in protein precipitated plasma. Analysis indicates that scanning quad DIA enables over an order of magnitude more specificity than a static quad operated with the same resolution and it was found that a quadrupole transmission window of approx 10 Da provided optimum identifications. Qualitative information was obtained by extracting lipid class information based on neutral loss or product ion extraction. Control, diabetic and obese human plasma samples were treated with isopropanol and centrifuged to precipitate proteins and the lipid-containing layer collected. 2DMS data were collected and differentially expressed lipid classes across the three conditions were quantified. The obtained results agreed with previous discovery studies and the expected changes in relation to disease and/or phenotype.

Novel Aspect

Targeted quantitation in lipidomics using a novel precursor quadrupole scanning based DIA method

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Quantitation of Proteomics Samples Using a Novel Scanning Quadrupole DIA Method

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Introduction

Targeted LC-MS assays are increasingly applied in the post-discovery proteomics area with an emphasis on validation. A novel DIA mode of operation, which provides both qualitative and quantitative information, has been developed for a tandem quadrupole/oa-time-of-flight (ToF) MS and applied to targeted proteomics experiments using transition extraction lists and compound library based approaches.

Method

NanoLC was performed with a Waters Nanoacquity UPLC equipped with a 75micron x 150mm column. The *m/z* isolation range of the quadrupole was continuously scanned with MS data acquired up to a frequency of 400 spectra/s. Alternate MS scans comprised precursor and CID product ions. The resulting 2D data, *m/z* (ToF) *vs. m/z* (quadrupole) were processed using Progenesis QIP and Skyline informatics.

Results

Quantitative proof-of-principle data were acquired by serially diluting a four protein digest mixture into a proteolytic *E.coli* digest and quantitative information obtained by using transition extraction lists. Analysis of the data indicates that scanning quad DIA enables over an order of magnitude more specificity than a static quad operated with the same resolution and it was found that a quadrupole transmission window of approx 20 - 30 Da provided optimum protein identifications.

Aliquots of control, diabetic and obese human plasma samples were digested with trypsin overnight. 2DMS data were collected and differentially expressed peptides and hence proteins across the three conditions were quantified. Results show that peptides from apolipoproteins exhibited the most significant changes in good agreement with expected changes in relation to disease and/or phenotype.

Novel Aspect

Targeted quantitation in proteomics using a novel precursor quadrupole scanning based DIA method

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A rapid and selective quan/qual characterization of a novel DIA method for proteomics analysis and its application to biomedical research

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In typical data independent acquisition (DIA) methods on Q-TOF instruments, the quadrupole mass filter or ion trap either operates in wide pass mode or in stepped mode with typical transmission windows in the range of 1-20Da. Here we describe a mode of DIA operation whereby a resolving quadrupole is scanned repetitively over alternating low and elevated energy scans.The m/z range of the quadrupole was continuously and repetitively scanned with data acquired using a ToF acquisition system capable of delivering 2000 ToF spectra / s. Alternate scans contain low energy data for precursor and high energy data for fragment ions. The resulting 2D data format can be processed using both commercial and open source software for identification / quantitative results.The effect of the scanning quadrupole transmission window has been investigated to assess qualitative performance. Tryptic digest standards were injected onto a LC system and separated using a 90 minute gradient. It was found that quadrupole transmission windows of 20-30 Da provided optimum protein identifications. Over 1,000 proteins were identified from a cytosolic E.coli digest standard (4% FDR). Additional evaluations of this methodology for qualitative and quantitative proteomic analyses will be made via the analyses of two disparate sample cohorts - characterization of synaptic proteomes as part of a study of developmental brain disorders, and differential analyses of protein:protein complexes of calcineurin (Aspergillus fumigatus) as a function of mechanism-of-action of several antifungal drugs.

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Lymph node metastasis of primary endometrial cancers: Associated proteins revealed by MALDI imaging

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The prediction of lymph node metastasis using clinic-pathological data and molecular information from endometrial cancers lacks accuracy and is therefore currently not routinely used in patient management. Consequently, although only a small percentage of patients with endometrial cancers suffer from metastasis, the majority undergo radical surgery including removal of pelvic lymph nodes. Upon analysis of publically available data and published research, we compiled a list of 60 proteins having the potential to display differential expression between primary endometrial cancers with versus those without lymph node metastasis. Using data dependent acquisition LC-ESI-MS/MS we were able to detect 23 of these proteins in endometrial cancers, and using data independent LC-ESI-MS/MS the differential expression of five of those proteins was observed. The localization of the differentially expressed proteins, was visualized using peptide MALDI MSI in whole tissue sections as well as tissue microarrays of 43 patients. The proteins identified were further validated by immunohistochemistry. Our data indicate that annexin A2 protein level is upregulated, whereas annexin A1 and α actinin 4 expression are downregulated in tumours with lymph node metastasis compared to those without lymphatic spread. Moreover, our analysis confirmed the potential of these markers, to be included in a statistical model for prediction of lymph node metastasis. The predictive model using highly ranked *m/z* values

identified by MALDI MSI showed significantly higher predictive accuracy than the model using immunohistochemistry data. In summary, using publicly available data and complementary proteomics approaches, we were able to improve the prediction model for lymph node metastasis in EC.

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Spatially-resolved metabolite quantification in the aging human lens

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To protect against oxidative-stress induced protein damage, the lens contains high levels of antioxidants, such as glutathione and ascorbic acid. The development of age-related cataract is thought to be related to changes in lens antioxidants and other metabolites in specific lens regions, and leads to the protein damage, insolubilisation and opacification that characterises lens cataract. In this study, lens small molecules and metabolites have been mapped in the aging human lens to define lens metabolome changes associated with normal lens aging, and a method to quantitatively map the distribution of predominant lens antioxidants developed using glutathione as a model.

Cryosections from human lenses ranging in age from 29 to 82, and lens homogenates spiked with different concentrations of heavy GSH, were coated with NEDC matrix and analysed simultaneously by a MALDI FTICR mass spectrometer (Bruker Solarix-XR 7T), in negative ion mode with MALDI IMS spatial resolution of 100µm. Data were imported into SCiLS Lab 2015b for analysis, while standard curves were plotted using Microsoft Excel.

Signal for glutathione was detected in lenses of all ages, in addition to human lens-specific UV filters. For example, 3-hydroxykynurenine glucoside (3-OHKG) was more abundant in the lens nucleus than the cortex, and signal intensity decreased with lens age. In contrast, a signal for putative lens fluorophore 3-OHKG-GSH was detected only in the old human lens nucleus. With age, glutathione signal declined, first in the nucleus, then in the cortex. For glutathione quantitation, a standard curve using heavy GSH was used to quantitate GSH signal levels in the human lens sections, which ranged from 5µmol/g in the lens cortex to 0.7µmol/g in the nucleus. This study highlights the metabolic changes that take place in the aging human lens, providing a baseline measure for age-related changes to be compared with metabolic changes associated with cataract formation.

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Improved reproducibility in MALDI imaging using a scanning laser beam

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MALDI imaging for the analysis of tissue is a technique for which many see potential clinical applications. To unlock this potential, the technique needs to fit into clinical workflows in regards to speed, reproducibility, reliability and ease-of-use. Some of these aspects have multiple influences that come from the sample itself or the handling thereof, the matrix application or the MALDI instrument itself.

As instruments with a continuously moving sample stage and a scanning laser beam are introduced, a significant improvement in scan speed can be achieved. Increasing the speed is often associated with decreasing spectral or image quality, but anecdotally many operators have reported an improvement in image quality. Here we measure and quantify this improvement.

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Boosting compound identification confidence by exploiting all HRAM spectral information: Integrating accurate mass, true isotopic pattern, in-source fragmentation, MS/MS fragmentation, and retention time

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Confident compound identifications is still one of the major bottlenecks in metabolomics. While there are ongoing efforts to refine the definitions and levels of metabolite identifications that were first proposed by the MSI initiative, it is clear that higher levels of identification confidence can be reached by joining accurate measurement technology, orthogonal molecular features, and sophisticated software tools. Here we present a single integrated software solution for pushing the confidence in identifications at different levels: molecular formula, compound class, structure, or verified targeted identification. This highly integrated functionality is implemented in a new version of the MetaboScape® software.

We could highly improve the quality of compound identifications in a study investigating the arginine biosynthesis in Corynebacterium glutamicum conducted by HRAM LC-QTOF non-targeted metabolomics. The integrated tools in MetaboScape were used to create annotations throughout increasing confidence levels: First, for all compound spectra molecular formulas were generated based on accurate masses, true isotopic patterns, and in-source fragmentation patterns, applying metabolomics-tailored rules and filters. Afterwards, public chemical databases were queried to find structural candidates for the generated molecular formulas of interesting features. Then, a customized analyte target list was applied to additionally exploit retention times of expected features. Lastly, MS/MS spectral library comparisons and in-silico fragmentations (MetFrag [1,2]) enabled to create and verify identifications based on MS/MS fragmentation patterns.

The outlined strategy will enable users to achieve highest confidence in compound identifications based on LC-HRAM-MS/MS spectral information using an integrated “turnkey” solution.

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2. Ruttkies C., et al.; Journal of Cheminformatics 2016, 8:3

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Unpicking the Unique Neutrophil Glycobiology in Cancer, Inflammation and Infection using Glycoanalytics

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Neutrophils are front-line immune cells critical for the innate immune system. Many aspects of the structure and function of the neutrophil glycoproteome remain unresolved. Enabled by technology advancements in glycomics and glycoproteomics, the intriguing protein *N*-glycosylation of human neutrophils was investigated in the context of cancer, inflammation and infection. A novel class of truncated *N*-glycoproteins, paucimannosidic proteins, was discovered in sputum from pathogen-infected human lungs (Venkatakrishnan et al., *Glycobiology*, 25(1):88, 2015). The monosaccharide compositions i.e. $Man_{1-3}GlcNAc_2Fuc_{0-1}$, the biosynthetic machinery involving maturation stage-specific β-hexosaminidase expression and the preferential location in azurophilic granules of neutrophils were demonstrated (Thaysen-Andersen et al., *J Biol Chem*, 290(14):8789, 2015). Importantly, these compartment- and inflammation-associated glyco-signatures were carried by intact bioactive proteins including cathepsin G, azurocidin and neutrophil elastase (Loke et al., *Biomolecules*, 5(3):1832, 2015). Glycomics-based studies on isolated neutrophil granules confirmed that paucimannosylation is enriched on azurophilic granules-resident proteins, but present also in other compartments. Paucimannosidic proteins were preferentially secreted, but not incorporated into the plasma membrane above constitutive levels, upon inoculation of isolated neutrophils with virulent *P. aeruginosa* thereby confirming granular mobility of paucimannosidic proteins. Preliminary data shows that isolated paucimannosidic proteins displayed affinities to mannose-binding lectin and bacteriostatic activities towards virulent *P. aeruginosa* supporting immune-related functions of paucimannosylation in activated human neutrophils. Interestingly, isolated neutrophils from Sandhoff disease patients displaying a *HEXB*$^{-/-}$ genotype showed reduced protein paucimannosylation relative to age-paired healthy donors confirming the importance of β-hexosaminidases in paucimannosidic protein biosynthesis. Finally, other immune and cancer cells were shown to also express paucimannosidic glycans indicating that these under-reported glycoproteins are integral to the immune system across cell types. In conclusion, we provide insights into the intriguing features of neutrophil glycobiology by expanding our knowledge of the structure, function and biosynthesis of the spatiotemporally-regulated protein paucimannosylation in the context of cancer, inflammation and infection.