1

Exploring alternative proteases and fragmentation methods for proteomics

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Proteome analysis heavily relies on a broad mixture of analytical techniques, starting from sample preparation, to separation and enrichment and last but certainly not in the least mass spectrometry. Through developments in these enabling technologies MS-based proteomics has matured and start to deliver biological relevant information. Still our measurements are still far from comprehensive, and the optimal matured workflow does not (yet) exist for proteomics. In my laboratory we try to explore alternative complementary methods to explore better the richness of the proteome
In more detail I will describe methods enabling 1) enhanced peptide coverage based on a combination of HCD and ETD that leads to improved analysis of phosphopeptides, MHC peptides [1] and protein disulfide bridges [2], 2) enhanced protein and proteome coverage, by using multiple proteases and 3) improved analysis of intact proteins and protein complexes enabling to visualize all proteoforms of a given protein at once and following kinase phosphorylation in real-time using top-down approaches [3]

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2.   Liu et al. Mol Cell Prot 13 (2014) 2776-2786
3.   Van den Waterbeemd et al. Angew Chemie 53 (2014) 9660-9664

2

Bulking up muscle proteomics: the exercise-regulated phosphoproteome and the control of muscle growth

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Exercise plays an essential physiological role in the regulation of acute and long-term muscle health, insulin sensitivity and whole body metabolic homeostasis. However, only a few signalling pathways and downstream substrates are known to trigger these beneficial effects. We performed the first mass spectrometry (MS)-based phosphoproteomic analysis of human skeletal muscle biopsies obtained from healthy male subjects before and after an acute bout of high-intensity bicycle exercise. We identified 1,004 phosphosites that were significantly regulated with acute exercise. More than 900 of these phosphosites have not previously been associated with exercise and the upstream kinase is unknown. Given the known therapeutic potential of the AMPK pathway, we performed two additional MS screens to specifically pinpoint novel AMPK substrates in the human muscle including: (1) global MS-based phosphoproteomic analysis of myotubes with pharmacological AMPK activation; and (2) a novel global AMPK *in vitro* kinase assay combined with targeted phosphopeptide quantification with data-independent acquisition MS. Integration of these data sets revealed a number of novel AMPK substrates including A-kinase anchor protein 1 (AKAP1) that was validated *in vivo*. Phosphosite mutations identified a novel role for AMPK-phosphorylation of AKAP1 in mitochondrial respiration. We next investigated the use of proteomics to identify substrates involved in the regulation of long-term muscle growth. Follistatin, a potent inhibitor of TGFbeta-signalling, regulates muscle hypertrophy through an incompletely understood mechanism. Proteomic analysis of mice muscle-specific and inducible adeno-associated virus (AAV)-mediated overexpression of follistatin was performed over a time-course. Numerous proteins not previously implicated in the regulation of muscle growth were identified including the E3-ubiquitin ligase, ASB2. AAV-mediated overexpression of ASB2 induced significant muscle atrophy highlighting a novel regulator of muscle mass. These data and developed methodology will serve as an invaluable resource for future studies of muscle physiology and the development of novel therapeutics.

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TWENTY YEARS OF THE PROTEOME

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On Monday the 5th of September in 1994, during the first Siena meeting (2D Electrophoresis – from protein maps to genomes), I presented the concept of the proteome and the term itself. Since that time, the field we now know as proteomics has expanded dramatically. Proteomic technology, and the new experimental paradigms that this technology supports, has revolutionised the way in which proteins can be studied in the challenging context of a cell or tissue. It has provided stunning insights into the diversity of proteins, their expression and many aspects of their function. This presentation will discuss 20 years of the proteome and of proteomics. It will discuss some major achievements of the field, some of the grand challenges that remain, and will speculate a little about what the future might hold.

Advent of Proteomics Discourse in Australia: The Lorne Proteomics Symposia

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This year marks the 20th anniversary of the Lorne Proteomics Symposium, an annual meeting of the Australasian Proteomics Society. From its humble beginnings, the APS meeting (for which it is now commonly referred to) has grown into an internationally recognized meeting for proteomics scientists to exchange ideas, start new collaborations, promote new technologies and to gather socially amongst friends and start up new friendships and collaborations on a global scale. The Lorne Proteomics Symposium of the APS is now considered a world-class meeting and has hosted well over 100 international guests over the last 20 years. The concept of the meeting was originally to provide a technology focused day prior to the Lorne Proteins Conference (a world class meeting in itself now in its 40th year. These "technology days" proved to be so successful, the meeting soon found itself expanding to include many aspects of protein identification and quantitation to also promote biological, clinical and agricultural applications of the technology. Lending from the phrase "proteomics" coined by Marc Wilkins for part of its name, this meeting now encompasses all aspects of proteome based studies and has become a fertile and important international meeting as part of the world wide proteome organization. We celebrate the 20th anniversary of the Lorne APS meeting.

The Hitchhiker's guide to Glycoproteomics and the human Immunoglobulome

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Glycosylation is well known to alter and fine-tune the functions of glycoproteins. A wide range of biological functions has been described such as conferring proteolytic resistance and mediating or inhibiting inter- and intracellular interactions [1]. As a matter of fact, protein glycosylation does not introduce a single, universally definable functionality. Thus detailed knowledge on the protein specific glycosylation and its site distribution is crucial for any systematic investigation of the functional aspects that protein glycosylation is playing on individual glycoproteins.

In that context mass spectrometry has become the method of choice for the identification and in depth primary structure characterisation of glycoconjugates. Nevertheless, it lacks the intrinsic ability to easily distinguish structural isomers of isobaric compounds, and the non-template based bio-synthesis of glycans as well as their non-linear structures pose additional challenges in glycan structure assignment by MS.

This presentation will give an overview on the peculiarities of protein glycosylation analysis and will focus in particular on advantages, challenges and limitations for in-depth glycoconjugate characterisation by MS. Synthetic glycopeptides, where both the peptide and glycan moieties can be adjusted, have become essentials tools for the systematic investigation, benchmarking and development of novel approaches for qualitative and quantitative glycopeptide analysis [2, 3]. In combination with orthogonal approaches for the analysis of the released N-glycans (porous graphitized carbon nanoLC ESI-MS/MS & CGE-LIF) and O-glycans (PGC nanoLC ESI-MS/MS) [4, 5], a detailed glycomics and glycoproteomics map of the entire human Immunoglobulome (IgA, sIgA, IgD, IgE, IgG and IgM) has been established, revealing the potential type II receptor sites present on human Immunoglobulins.

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Development of LongBow TM - an integrated system for high throughput glycomics to support biopharmaceutical realisation and clinical diagnostics

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'I have umpteen thousand samples to analyse. How can I get reliable, detailed, quantitative maps of the complex glycosylation for each of them without spending a great deal of time and vast sums of money?'

This talk gives an overview of LongBow - an integrated glycomics platform being developed to address this problem - which is becoming increasingly important in two areas of modern healthcare:

a. Biopharmaceutical realisation . Glycomics studies are essential throughout the development and biomanufacturing of most glycoprotein therapeutics because changes in the glycosylation can greatly influence the safety and efficacy of the drugs in patients.

b. Clinical diagnostics . Glycans play key roles in many critical biological processes and changes in glycosylation patterns of serum glycoproteins are potential early-warning biomarkers for a number of chronic diseases. These include cancers and serious inflammatory conditions such as inflammatory bowel disease (IBD).In both cases, we are interested in monitoring the (often subtle) changes in glycosylation patterns that correlate either with altered clinical performance (for the drugs) or disease progress in the patient. However, determining glycosylation patterns is still very challenging because of the complexity and heterogeneity of glycan populations. The complexity arises from the diversity in glycan monomer types and the ways they can be assembled with branched topologies. Regarding heterogeneity, both biopharmaceuticals and biological samples may be bear tens to hundreds of different types of glycans. For full glycomics studies we need to identify the complete structure of each glycan and determine its relative or absolute abundance to a high level of accuracy. Currently, there is no single analytical platform that can do this reliably, quickly and affordably. LongBow is being designed as a first step towards achieving this and implements concepts developed in the HighGlycan programme. This is a five year, €6m, EU-FP7 funded collaborative research project involving nine glycoscience organisations across Europe.The main topics covered in this overview will be:

1. Overall design of LongBow, how the components fit together, flexibility for chemistry and instrumentation
2. Types of samples: biopharmaceuticals, plasma, serum glycoproteins, cells and tissues
3. Instrumentation - robotics and orthogonal analytics (UHPLC, MS, LC/MS and xCE)
2. Sample processing workflows for glycopeptides, N-glycans, O-glycans, sialic acids and neutral monosaccharides
3. Integration of modules, performance and validation
4. VTag TM system for glycopeptide analysis by UHPLC and MS
5. Procainamide system for glycan analysis by UHPLC and MS
6. Applications: How LongBow is being used at Ludger for biopharmaceutical glycoprofiling to support drug comparability studies and regulatory submissions and for medical glycomics for stratification of patients with chronic inflammatory conditions
7. Transfer of LongBow technology for glycomics studies in partner labs
8. Advantages and limitations of LongBow, future improvements.

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UniCarbKB: a glycobioinformatics infrastructure for data discovery

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In partnership with leading international research groups we are involved with the development of the UniCarb KnowledgeBase (UniCarbKB); an effort to develop and provide an informatic framework for the storage of high-quality data collections including informative meta-data and annotated experimental datasets.

UniCarbKB is a new database that aids the exploration of glycoproteins pertinent to current research strategies that strives to be a comprehensive resource. To sufficiently address research questions we aim to: 1) build on the knowledge originating from GlycoSuiteDB by carefully including selected and filtered glycoprotein data; 2) organise data to enable user-friendly interaction and querying by adopting standardisation and ontology guidelines; and 3) build a platform that will support the inclusion of new data mining tools and connect disparate resources. Here, we shall demonstrate the functionality of UniCarbKB and our strategy to increase the content and value of data provided by UniCarbKB, and our efforts to build and provide open APIs for the glycobioinformatic community.

Glycobioinformatics databases and tools are co-operatively adopting semantic technologies for managing data content, called GlycoRDF. The availability of GlycoRDF and access to RDFized databases is opening new and exciting avenues for connecting and interrogating large volumes of publicly accessible data. Examples of the capability of UniCarbKB-RDF for exploring and correlating glycomics structural and experimental data will be presented. Furthermore, the utility of semantic technologies to connect glycan-related knowledge bases with other omics resources (UniProtKB and NeXtProt) to enhance data discovery and inference of protein and glycan biological function will be highlighted.

The development of UniCarbKB and supporting technologies continues to provide a new perspective on glycobioinformatics, which extends access to high-quality annotations with interfaces to supporting analytical data sets. The initiative will be driven as a community endeavour to promote data sharing to ensure its future development and growth. Especially, our efforts to align data capture with the GlycoRDF, MIRAGE and international glycan repository initiatives.

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N- and O-glycomics of FFPE tissues from histopathological slides

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Protein glycosylation is a ubiquitous post-translation modification well known to functionally influence many biological processes such as immune modulation, cell-cell interactions or signal transduction. The composition of a cell's glycome is dependant of numerous factors such as age, health or disease state, and several examples successfully demonstrated the applicability of individual glycosylation alterations as specific disease markers. This makes glycosylation a prime target for biomarker discovery, nevertheless the availability of well-defined clinical specimens in sufficient numbers often represents a serious obstacle in glyco-biomarker analyses. The possibility to use formalin-fixed paraffin-embedded (FFPE) clinical specimens presents an attractive alternative for glyco-biomarker research given that any glyco-epitopes remain unaltered and sufficient glycan amounts can be obtained from conventional FFPE tissue sections frequently used by pathologists.

Here we present an approach for the in-depth glycomic profiling of both, N-and O-glycans starting from single FFPE tissue sections as thin as 3 μm. Porous graphitized carbon (PGC) nanoLC ESI-MS/MS provides excellent isomer separation, semi-automated structure identification via spectral matching and relative quantitation of individual structures. Unstained as well as hematoxylin and eosin (HE) stained FFPE tissue samples derived from routinely taken clinical preparations were analysed and compared to their corresponding frozen counterparts to identify any potential FFPE and staining induced alterations on the N- and O-glycome. Furthermore, N- and O-glycans could successfully be retrieved from cells isolated by laser micro dissection from FFPE sections (ranging from 10000 down to 1000 cells). Overall around 90 individual glycan structures including structural isomers were identified from human hepatocellular carcinoma cells and non-tumour liver tissue. Major glyco-epitopes containing neuraminic acid and/or fucose were not affected in their structural integrity. These results emphasise the potential provided by FFPE tissue slides as a valuable source for glyco-biomarker studies from clinical specimens.

9

Tracking protein turnover in plants: a new frontier in understanding the cost of proteins to plant growth

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Shotgun approaches or targeted SRM analyses have dominated proteome studies as tools to find changes in the proteome. These approaches, however, focus just on the proteins that are changing in abundance in order to find biological insights. They also require statistically significant changes in the total accumulated protein pool size in order to identify that 'anything has occurred'. Analysing protein synthesis and degradation rates with progressive stable isotope labelling provides a new window on the control of protein abundance. With this approach we can determine the 'relative age' of the proteins that we see and define the energetic effort employed by the cell to build or maintain particular activities. We are using progressive ^{15}N labelling of plant cells from nitrate and ammonia salts and modelling incorporation fits, to calculate the rate at which proteins which are often static in abundance in the proteome are turning over. We have developed pipelines to undertake these studies in plant cells, plant leaves and in whole plants through the use of hydroponics. Projects assessing the impact of leaf age, phosphate limitation and groundwater salinity on protein turnover changes in plants will be discussed. Through combining such labelling with separation of protein complex and subcomplexes by native electrophoresis, we can observe the *in vivo* turnover rate of assembly intermediates of protein complexes. Combined there approaches provide new avenues for peptide mass spectrometry to provide answers to a wide range of questions in plant biology, and allows researchers to assess the cost of environmental factors on protein turnover and plant growth efficiency.

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Proteomic Analysis of Abiotic Stress in Plants

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Proteomic studies in plants including rice (*Oryza sativa*) and grapes (*Vitis vinifera*) have become increasingly prevalent in recent years now that complete genome sequences are available for both species. In our laboratory we have carried out a number of different studies involving analysis of differential protein expression in both plants and cell cultures, induced by exposure to different abiotic stress conditions. This has included detailed analysis of the response of grape cells to a wide range of temperature stresses, identification of proteins affected by changes in day length in riverbank grapes, and detailed quantitative analysis of temperature and drought stress in rice. This work was all performed using shotgun proteomics analysis with nanoLC-MS/MS, coupled with calculation and measurement of normalised spectral abundance factors using software developed in-house. This analytical pipeline enables the identification and quantitation of thousands of proteins simultaneously without the need for metabolic or chemical isotopic labelling. In this presentation we will present some highlights from these studies, focusing on the commonalities of the biological responses observed in these and other studies. We will also discuss statistical validation of protein expression changes, including recent work on the development of a procedure for estimation of false discovery at the protein quantitation level.

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Identification of N-linked glycosylation sites on plant glycoproteins

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N-glycosylation of plant proteins has been shown to be important for catalytic activity, folding, subcellular location and secretion, as well as in plant pathogen interactions[1]. At the modification site there is (micro)heterogeneity of the glycans attached, making it difficult to detect in routine proteomics studies and the labile nature of the glycan hinders the analysis by mass spectrometry. We describe a method for the enrichment of N-linked glycopeptides from plants, followed by LC-MS/MS analysis using higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) for peptide identification, glycan site attachment and the type of glycans attached.

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A Top-Down Proteomic Approach for Understanding Multiple Sclerosis

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Multiple Sclerosis (MS) is conventionally thought to be initiated by an autoimmune T cell response. However, there is an alternate hypothesis that MS may begin as a slow, progressive (i.e. clinically undetected) degeneration of oligodendrocytes [1-3]. Currently the molecular mechanism(s) behind such a degeneration are poorly understood. To address this we have initiated a systems-wide analysis of an animal model of oligodendrocyte degeneration: the cuprizone model. We have undertaken a top-down analytical approach to investigate the breadth of proteomic alterations that cuprizone treatment has on the cortex, skeletal muscle, spleen, and peripheral blood mononuclear cells. Using 2-dimensional gel electrophoresis, deep imaging, and mass spectrometry we have identified 43 protein species as uniquely present or absent in the cuprizone treatment group. Protein species identified in the cortex are potentially linked to primary oligodendrocyte degeneration and secondary axonal damage. Furthermore, we identified alterations in the proteomes of skeletal muscle, spleen, and peripheral blood mononuclear cells following cuprizone treatment; arginase-1 was a notable hit in the spleen and has previously been linked to T cell suppression in an animal autoimmune model of MS [4-6]. A further notable finding was the absence of protein disulphide isomerase subunits in the spleen and peripheral blood mononuclear cells of the cuprizone treatment group; this may further contribute to impaired MHC class I assembly leading to reduced antigen presentation and potentially contributing to the lack of T cell response in this model [7]. Overall, the results of this study suggest that findings from the cuprizone model and likely others need to be carefully considered relative to clinical MS.

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Serum glycoprotein biomarkers for oesophageal adenocarcinoma

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While the incidence of most cancers are now steadying or declining, oesophageal adenocarcinoma (EAC) continues an upward trend. The rapid increase in EAC is not due to improved diagnosis, but likely attributed to the increased prevalence of risk factors of obesity and gastro-esophageal reflux. Despite aggressive treatment, the survival rate for EAC is low at 9-24% five years post diagnosis. The precursor condition, Barrett's oesophagus (BE), affects 0.2-2% of the adult population and increases EAC risk 30-100 fold. However, due to the low conversion rate of BE to EAC, studies indicate that current endoscopic screening programs may not be beneficial. Furthermore, a significant proportion of EAC patients do not have prior BE diagnosis, hence there is an urgent need for better detection of EAC. Our goal is to develop blood biomarker panels that can be used to screen at-risk patients, with positive or suspicious results triggering follow-on endoscopic screening.

We focused on alterations in circulatory protein glycosylation, using a panel of 20 lectins to enrich serum glycoproteins based on their glycan structures[1, 2]. Serum samples from healthy, BE and EAC patients (n=29) were analyzed by lectin magnetic bead

array (LeMBA)[1, 2]-coupled discovery proteomics using QTOF, followed by targeted proteomics using MRM-MS (n=61). Data analysis was performed using a customized database and analysis package "GlycoSelector" incorporating sparse Partial Least Squares-Discriminant Analysis (sPLS-DA)[3].

We have identified a ranked list of glycoprotein biomarkers that distinguish a) EAC from BE and b) EAC from healthy phenotypes. Selected biomarkers were further validated using an orthogonal technique, immunoblotting. A multivariate panel achieved area under the receiver operating curve (AUROC) over 0.9, indicative of high diagnostic value. Continuing work will evaluate clinical performance of the EAC biomarker panel in a large independent patient cohort.

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Revealing metabolic actions of novel compounds against African trypanosomes by high resolution mass spectrometry

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Trypanosoma brucei causes human African trypanosomiasis (HAT), which is transmitted by a bite of the Testse fly. This disease causes significant disability and is fatal for around ten thousand people every year. The armory of medication for HAT is outdated and the development of resistance, high toxicity and treatment costs are ongoing problems. High throughput screening of approximately 87,000 compounds against *T. brucei* identified a novel compound 3-(Oxazolo[4,5-b]pyridine-2-yl)anilide (OXPA) which shows high activity against this organism and minimal toxicity to mammalian cells. The aim of this study was to determine the biochemical actions of this compound in *T. brucei*. Metabolomics with HILIC/RP-chromatography in combination with high resolution mass spectrometry was applied to *in vitro* cell cultures of *T. brucei* treated with this novel compound. The investigation of polar metabolites with untargeted HILIC metabolomics analysis suggested that this potential drug perturbed carnitine uptake, trypanosome-specific NADH-fumarate reductase, trans-sialidase and 6-phosphoglucolactonase. The most interesting finding was the accumulation of several ceramides, which play an important role in cell signaling and the synthesis of sphingolipids in the parasite. In order to understand changes in the lipidome of the parasite in more detail a new lipidomics method was developed which enables the identification of hundreds of lipids with high throughput and sufficient reproducibility. The application of this novel method in an untargeted analysis identified 600 lipids in the parasite indicating that the lipidome of *T. brucei* mainly consists of glycerophospholipids, sphingolipids and fatty acyls. Interestingly, this analysis confirmed extensive ceramide accumulation in the parasite due to treatment with the novel compound OXPA.

This study strongly suggests that OXPA interacts with the sphingolipid pathway in *T. brucei*, most likely by inhibiting the parasitic sphingolipid synthase, which has been validated as a potential drug target in previous studies.

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Harbouring a killer: a proteomic approach to comparing host responses to Hendra virus

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Bats are a major source of zoonotic pathogens, harbouring over 60 different viruses including Ebola, SARS, MERS and Hendra (HeV), many of which pose a serious threat to human and animal health. HeV is an emerging zoonotic virus currently only found in Australia. It causes respiratory and neurological diseases in horses and humans and is characterised by high morbidity and mortality. However in its natural fruit bat host (*Pteropus* spp.), HeV does not seem to cause any symptoms of illness, with high titres of virus shed in bat excretions. Currently, there is no vaccine or treatment against HeV for humans. We hypothesise that comparative studies of the bat and human immune response to HeV may provide valuable insight into deciphering the elements of an effective anti-viral strategy.

In this study, two hosts, bats (*Pteropus alecto*) and ferrets (*Mustela putorius furo*) (human disease model) were challenged with HeV and tissues were extracted at several time points. Tissues were homogenised and analysed by mass spectrometry (LC-MS/MS). Identified proteins were then quantified using label free approaches. Preliminary data has highlighted a difference between the bat and ferret host response to HeV infection. In bats, there was increased expression of proteins involved in innate immune responses such as the type I interferon signalling pathway. However, in ferrets, there was a strong skewing towards activation of adaptive immune responses including proteins involved in antigen processing and presentation. In both models, there was an overlap in the induction of proteins involved in the complement system. These data provide candidate pathways for further examination using targeted mass spectrometry and orthogonal techniques. Validation using these sensitive and quantitative approaches will further elucidate the mechanisms by which bats are able to control viral infection while humans succumb to disease. Ultimately, it will also address the question of whether bats tolerate virus due to superior innate immunity or whether a propensity towards immunopathology in humans contributes to the mortality of HeV.

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Facing the fats: Can mass spectrometry unmask the true structural diversity of the lipidome?

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Lipids play pivotal and diverse roles in cellular function, ranging from structuring cell membranes to energy storage and signaling. Understanding the full range of the biological functions of lipids, and the associated mechanisms, requires a detailed understanding of the diverse array of molecular structures that constitute the lipidome.[1] Recent advances in mass spectrometry – notably the increases in sensitivity and resolving power – have revealed the staggering molecular complexity of lipids in biological extracts. Elucidating the molecular structure of these lipids within such rich mixtures however, represents a formidable challenge to conventional mass spectrometry. The recent combination of collision-induced dissociation (CID) with ozone-induced dissociation (OzID) has been shown to be a powerful approach to providing a near-complete structure of complex lipids including the assignment of position(s) of unsaturation and relative acyl chain positions in glycerolipids.[2] Importantly, these novel ion activation strategies identify the presence of isomeric lipids within complex extracts and examples from investigations of lipid extracts from humans and insects indicate functional significance of specific isomeric forms.

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Identification of *Plasmodium berghei* markers of infection in the arthropod host by TLC-MALDI coupled to Imaging Mass Spectrometry

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Thin Layer Chromatography (TLC) coupled to Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS) offers the ability to couple a rapid and versatile separation technique with routine MALDI MS analysis providing an effective strategy for the analysis of lipid mixtures. Due to the varying detectability's of individual lipid classes when using a MALDI Imaging Mass Spectrometry (MALDI-IMS) approach, prior separation using TLC offers advantages including detection of either low abundance or suppressed ions coupled with the ability to analyse multiple samples (e.g. control and infected) at the same time. The complement of lipids extracted from excised midguts of control and mosquitoes infected with GFP labelled *Plasmodium berghei* ANKA, were separated by TLC using optimised separation and matrix application conditions prior to MALDI Fourier Transform MS (FTMS) analysis. Using this approach we putatively identified a phosphatidylethanolamine (PE(38:5)) as a marker of the infection status. Subsequent tissue imaging using MALDI-FTMS and classification using Receiver Operating Characteristic (ROC) curve analysis of control and infected mosquitoes tissue sections confirmed the PE(38:5) to be collocated with the site of infection within the midgut. Further, a number of polyunsaturated fatty acids corresponding to eicosapentaenoic acid and eicosatetraenoic acid, implicated in both parasite development and host immune response, were found to be highly abundant in infected tissues sections.

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Lipidomics is providing new strategies for risk assessment and therapeutic intervention in cardiovascular disease

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The metabolic syndrome incorporating obesity, hypertension, dyslipidemia and elevated plasma glucose has reached epidemic proportions in many countries leading to an increased prevalence of type 2 diabetes (T2D) and cardiovascular disease (CVD). Dyslipidemia, as assessed by standard measures (raised plasma triglycerides and LDL-cholesterol, and decreased HDL-cholesterol) is an independent risk factor for T2D and CVD. However, current risk prediction algorithms have limited accuracy. Further to this, the mechanistic links between dyslipidemia, T2D and CVD are complex and not well understood. Lipidomics presents a new set of tools to address these issues.

We have developed a targeted lipidomics platform using liquid chromatography electrospray ionization-tandem mass spectrometry to profile 300-400 lipids from 10 mL plasma. We have applied this technology to multiple clinical and population based cohorts to define the plasma lipid profiles associated with T2D and CVD and evaluate the potential application of these profiles to diagnose, assess disease risk and inform on disease pathogenesis.

Regression analysis adjusting for covariates (age, sex, systolic blood pressure and obesity) identified multiple lipid species that were significantly associated with prevalent and incident T2D or CVD. Multivariate analysis incorporating unsupervised feature correlation minimization and reliefF feature selection was employed to create and test multivariate classification and risk

assessment models incorporating different numbers of lipids and other risk factors.   Models incorporating lipids performed better than models based solely on traditional risk factors.

Plasmalogens, a class of lipids with anti-oxidative and anti-inflammatory properties, were negatively associated with prevalent and incident CVD. We subsequently investigated the potential of batyl alcohol (BA, metabolic precursor to plasmalogen) to modulate plasmalogen levels and attenuate the development of atherosclerosis in ApoE$^{-/-}$ and ApoE$^{-/-}$GpX1$^{-/-}$ mice fed a high-fat diet. The mice without BA developed extensive atherosclerotic plaques throughout the aorta. This was reduced by 70% ($P<0.001$) in the BA-treated mice.

Plasma lipid profiling can provide insight into disease pathogenesis and may contribute to a new approach to risk stratification and therapeutic intervention in T2D and CVD.

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Probing microbial pathogen interactions and metabolism using metabolomics

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Protozoan parasites are an evolutionarily diverse group of single-celled eukaryotes that cause a number of important diseases, such as malaria, toxoplasmosis and leishmaniasis, that collectively affect hundreds of millions of people world-wide. There are no vaccines for any of these diseases and current drug therapies are extremely limited and/or being undermined by the emergence of drug-resistant parasite lines, and there is a urgent need to identify new drug targets. While metabolic enzymes are high priority drug targets, our current understanding of the cellular metabolism and physiology of intracellular parasite stages is limited. In particular, while genome-wide annotations have provided broad insights into the metabolic potential of these pathogens, the majority of protein-encoding genes in these genomes (60-70%) have no known function. Furthermore, attempts to infer stage-specific changes in metabolism from transcriptional or proteomic analyses have also been hampered by the reliance of these organisms on post-translational responses. In order to identify metabolic pathways that are active in pathogenic stages we have developed a comprehensive suite of MS-based metabolomic approaches for characterizing parasite metabolism in situ. In this talk, examples will be provided of how MS-based metabolite profiling coupled with ^{13}C-stable isotope labelling approaches have revealed novel aspects of parasite metabolism that are essential for intracellular survival. We have also developed new approaches for measuring parasite and host responses in infected animal tissues using heavy water ($^{2}H_2O$) labelling and high resolution FTICR-imaging mass spectrometry. The latter approaches are being used to identify metabolically distinct populations of pathogens in tissues that may be differentially susceptible to anti-parasite drugs.

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Analysis of large proteomics datasets: pitfalls, challenges and solutions

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Since it is becoming feasible to collect shotgun proteomics data on the scale of the whole human genome it is crucial that computational workflows for the identification and quantification of peptides and proteins are equipped for dealing with mass spectral datasets of enormous sizes. The challenges are two-fold: 1) the requirements for the computational efficiency are daunting including demands for high degree of parallelization and efficient I/O. 2) Prescriptions for restricting the false discovery rate for peptides and proteins need to be utilized in order to ensure validity of the results. We show how these and other crucial problems are solved in the MaxQuant software and present examples of its application to several large-scale proteomics datasets.

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Data Independent Acquisition – The Next Revolution in Proteomics

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As the field of proteomics research has evolved over the past ten years, from identification focused experiments to today's highly quantitative strategies, different techniques have been developed to meet the workflow needs. Most recently, SWATH™ Acquisition, a data independent acquisition workflow, has been developed that increases the comprehensiveness of data collection while maintaining high quantitative reproducibility. Now, thousands of proteins can be profiled across large sample sets with high data completeness and quantitative accuracy approaching that of the gold standard MRM approach. In DIA, larger width Q1 windows are stepped across the mass range in an LC timescale, transmitting populations of peptides for fragmentation, and high resolution MS and MS/MS spectra are acquired. Post-acquisition, MS/MS data is interrogated using spectral libraries to generate quantitative XICs, providing MRM-like data for better specificity and quantitative accuracy. Significant progress has been made in understanding and improving the analytical attributes of this DIA technique, using key sample types. For a new technique to have impact on biomarker research however, it must be routinely implemented across multiple labs. Progress to date on the use of DIA workflows for biomarker research will be discussed.

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Mass spectrometry imaging of tryptic peptides: towards cellular resolution in fresh-frozen and FFPE tissue

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The direct detection and identification of intact proteins in MALDI imaging remains a challenging task due to limited sensitivity and mass range. Here we present new approaches for on-tissue tryptic digestion of proteins. We focus on optimizing the spatial resolution and reliability of peptide identification. We also present strategies for data processing based on the common data format imzML.

Trypsin solution was deposited in several cycles on tissue with a spraying device. An atmospheric pressure matrix assisted laser desorption (AP-MALDI) ion source coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific GmbH, Bremen) was used for imaging experiments. Tryptic peptides were identified by matching imaged m/z peaks to peptides which were identified in complementary LC-MS/MS measurements of an adjacent tissue sections. All MS measurements were based on accurate mass (< 3 ppm RMS).

A coronal mouse brain section was measured at 50 μm pixel size. Peptide peaks were detected on tissue with a mass resolution of R=80000 (@m/z700). This measurement reveals detailed histological structures such as the ependyma (consisting of a single cell layer) which is clearly defined by several identified peptides. Highly reliable information about protein distribution was also obtained for clinical human tissue originating from brain and gastric cancer biopsies. This data is used to investigate intratumor heterogeneity on a molecular level. A whole body section of an infant mouse was imaged at 50 μm pixel size. Strategies for processing this 40 GB data set by conversion to the common data format imzML will be discussed.

A coronal mouse brain section was imaged at a pixel size of 25 μm. The resulting ion images of tryptic peptides showed excellent correlation with myelin and H&E staining. Peptide peaks were detected on tissue with a mass resolution of R=40000 (@m/z700). The sensitivity could be significantly improved compared to previous experiments and about 150 tryptic peptides which show a clear spatial distribution were identified.

Initial results for formalin-fixed paraffin-embedded tissue will be discussed. Different protocols for deparaffinization and antigen retrieval were evaluated. Tryptic peptides were detected with accurate mass at 35 μm pixel size in mouse brain tissue.

Some of the datasets discussed here were up 40 GB. This requires efficient ways for data processing. An example on how to use the common data format imzML in order to make use of multiple software tools is discussed.

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2-D Metalloprotein maps: application of quantitative metal and protein proteomics to define the metalloproteome of the human brain.

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Despite the importance of metal cofactors, techniques to measure metal status in a proteomic fashion are not readily available. One major challenge for metalloproteomics is determining the identity of the metalloprotein. Thus, we have been pursuing the development of tools that will allow the direct identification and quantitation of individual metalloproteins from biological samples. The tools we have developed allow for comparative metalloproteomics and provide a metalloprotein map that is reminiscent of 2D polyacrylamide gels. The ability to resolve and measure metalloenzymes in detail have practical implications for a variety of research areas including neurodegeneration, cancer, aging, heavy metal toxicity and therapeutic development. Here we present metalloprotein maps for Cu, Fe, Zn, Mn, Co, Ni and P from human brain tissue. The combination of multidimensional chromatography and traditional proteomics has provided one of the first in-depth looks at the human metallproteome and has resulted in the discovery of novel metalloproteins. The future development and application of this technique promises to yield important insights into the role of metals in biological and pathological processes.

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The use of Axial Spatial Distribution Focussing for the generation of High Resolution and High Energy MALDI tandem mass spectra

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The curved field reflectron has been in existence for nearly two decades since it was introduced by Cotter and Cornish. For several years the CFR was used in the only commercially available instrument capable of MALDI TOF MS/MS without scanning the reflectron. Seamless PSD provided advantages in terms of both sensitivity and speed of acquisition. However, the key advantage of the CFR over conventional linear field reflectrons, even those used in TOF/TOF instruments has been the ability to produce MS/MS at the full extraction energy. This is because there is no need with the CFR to re-accelerate the ions after the first TOF. So that, not only is very high sensitivity achieved for MS/MS but also that true high-energy CID is possible with lab collision energies of 20keV or more.

However, even when used in MALDI TOF instruments with in excess of 20,000 mass resolutions for MS, the mass resolution using the CFR for MS/MS was limited to isotopic resolution up to about 1000 Da. This has been known for a long time to be due to the increase in the laser intensity required to produce the meta-stable ions required for MS/MS (even with high-energy CID) which is much higher than the threshold for MALDI TOF MS. However, until recently, the effect had not been understood or quantified. More importantly, the reduced mass resolution has meant that the CFR instruments were not able to match the ultimate performance of other TOF/TOF instruments.

In this paper, we present the basic theory behind Axial Spatial Distribution Focussing (ASDF) as well as its applications for various structural elucidations and applications problem. Lipids fragmentations data showing C-C backbone fragmentation as well as high energy tandem Mass spectra of Peptides allowing differentiation of isomeric species will be presented. With the technique of Axial Spatial Distribution Focussing in combination with the Curved-Field Reflectron, fragment mass resolutions in excess of 10,000 can be achieved for MS/MS of meta-stable ions and CID at lab energy of 20keV. By using ASDF, the Curved-Field Reflectron is again capable of defining the state of the art for MALDI TOF-MS/MS and can really be said to have come of age.

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Proteomic Determinants of Metastasis Formation in Pancreatic Adenocarcinoma

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Pancreatic cancer is currently the fourth most common internal cancer worldwide. With more than 200,000 reported cases annually, its mortality is almost equal to the incidence rate. Pancreatic cancer is an aggressive malignancy with one of the worst outcomes of all cancers and has a very poor prognosis: with a 1-year survival of less than 25%, and once the tumors have metastasized, a five year survival of less than 5%. In our study, we aim to investigate the key proteins from the primary tumor of pancreatic ductal adenocarcinoma (PDAC) that drives metastatic spread. Quantitative profiling of proteins using dimethyl labeling followed by 2D-LCMS/MS analysis of five primary tumours from patients with observed lymph node metastases (TNM classification=N1) were compared against five primary tumours from patients with no lymph node metastases (TNM classification=N0). We have identified and quantified a total of 2451 proteins in at least 3 of 5 patients, of which 105 proteins displayed altered levels in N0 versus N1 (p < 0.1, delta fold change >0.5). For selected proteins, the findings were validated by immunohistochemistry and Western blotting. Our data revealed a number of proteins that had already been related to cancer metastasis and tumour cell invasion such as dermatopontin, biglycan, activated leukocyte cell adhesion molecule (ALCAM), but also other potential new prognostic biomarkers such as DNA binding protein A and guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2. Furthermore, our data indicate a number of proteins which were related to immune responses and tumour microenvironment. Taken together, the preliminary findings from this study shed lights on novel candidates, which may play pivotal roles in cancer cell invasiveness and metastatic spread in pancreatic cancer.

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Interactome profiling of the human histone deacetylases: Insights into the stability and regulation of chromatin remodeling complexes

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Histone deacetylases (HDACs) are a family of 11 essential transcriptional regulatory enzymes. HDAC dysfunction contributes to many human disease states, including cancers and viral infections. Indeed, small molecule HDAC inhibitors are in clinical trials for treatment of cutaneous T-cell lymphomas. However, these inhibitors are relatively non-selective, and the functions and interactions of many HDACs remain poorly understood. A systematic characterization of unique and shared protein complexes among HDAC family members would provide insight into HDAC-specific functions and improved targets for therapeutic intervention. Towards these goals, we integrated fluorescence microscopy, immunoaffinity purifications, quantitative mass spectrometry, functional network analysis, and improved bioinformatics tools to build the first global protein interaction network for all eleven human HDACs in T-cells. Through optimization of label-free SAINT modeling of interaction specificity, we detected many well-known HDAC interactions as well as 200 previously unreported interactions. For instance, we identified deleted in breast cancer 1 (DBC1), a protein associated with tumor progression and known to inhibit HDAC3 and SIRT1. Our finding that DBC1 also interacts with HDAC5 and HDAC9 suggests a broader function, which we further characterized using molecular biology and biochemistry approaches. Apart from HDACs' transcriptional roles, functional interaction profiling of novel interactions highlighted emerging roles in spliceosomal snRNP assembly and cell cycle regulation. To provide a deeper functional understanding of HDAC regulation, we designed a hybrid approach integrating label-free and isotope-labeling quantification to profile relative interaction stability across HDAC protein complexes. Interestingly, HDAC1 interactions were either highly stable within chromatin remodeling complexes or fast-exchanging associations with DNA-binding proteins and transcription factors. Collectively, our work demonstrates the effectiveness of an integrative proteomics approach in extrapolating protein functions based on interaction networks. Our global HDAC interaction dataset led to the identification of new HDAC functions and provides a rich resource for investigating HDACs in health and disease.

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Empirical inference of topology and plasticity in cancer signalling networks

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Signalling pathways driven by the activity of lipid and protein kinases regulate key cell biological functions. Such pathways form complex networks of biochemical reactions that decode and integrate a multitude of inputs from the cell environment in order to produce an appropriate response. The topology of signalling networks has been inferred from experimental data collated from different studies using computational methods. As a complementary approach to network biology, this presentation will discuss our efforts in using empirical data (from MS-based phosphoproteomics) to infer signalling network topology de novo and without

a preconception of how such networks may be wired in specific cell types. Studies in which this approach was used to investigate network plasticity in cancer will also be presented

Proteomic profiling of activity-dependent presynaptic signalling

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Protein phosphorylation and dephosphorylation is a crucial fast signalling mechanism following depolarisation of presynaptic nerve terminals. The strength of connections in neural circuits is adaptable, depends on brain activity and underlies learning and memory. Depolarisation of axons and presynaptic terminals causes neurotransmitter filled synaptic vesicles to fuse with the membrane and release neurotransmitter into the synaptic cleft. The the amount of neurotransmitter released can be regulated by a range of different biological processes directly and indirectly related to the cycle of synaptic vesicle fusion and biogenesis. In the past, 32P metabolic labelling has provided a view of how presynaptic protein machinery engages with activity-dependent signalling. We have used chemical depolarisation of isolated presynaptic nerve terminals to profile presynaptic signalling and compared this to 32P metabolic labelling. Peptide derivatisation by iTRAQ or dimethylation with isotopes of different mass allowed quantification of phosphorylation levels over time. We have shown that the type and level of stimulus determines the presynaptic response and have identified regulators of the synaptic vesicle cycle and other biological processes as targets for activity dependent phospho-signalling.

The Prophetic Spermatozoa. How Male Infertility is Predicting the Health and Life Expectancy in Men.

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Male infertility is a very common condition, with reports suggesting that one in 15 men of reproductive age is affected[1]. The diagnosis of male-factor infertility is difficult and involves discounting female infertility through hormone measurements, pelvic examination and invasive laparoscopy. A semen profile analysis can suggest male infertility, if sperm counts are <15-20 million/ml, or <50% of sperm possess forward progressive motility (and < 25% rapidly progressive sperm) or <4% good morphology sperm. However, for many couples (20-30%), infertility remains largely unexplained.

Large scale cohort reports have shown that infertile men on average are dying younger than their fertile counterparts. In addition, infertile men on the whole demonstrate three times the average rates of cancer compared to the general population[2]. As such, it appears that spermatozoa may give a "prophetic" insight into the overall health of men with a current leading hypothesis suggesting that sperm cells may act as a "canary in the coalmine" for the future prediction of men's health[2].

As such, we have used quantitative proteomics analysis to compare spermatozoa taken from healthy, fertile individuals and compared the proteome to that of an infertile male. Several proteins were found to be altered, including, the sperm specific protein, Outer Dense Fibre 1, which was virtually absent from the gametes of the infertile male. Of particular note however, the epigenetic regulation of histones was shown to be very different in the infertile sample, compared to the fertile sample. This suggests that erroneous post-translational modifications on Histones within infertile men may represent the first insight into not only male infertility, but also may explain the link between infertile gametes and the future prediction of men's health.

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Using novel data independent acquisition methods to obtain characterization of a biological pathway

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Publish consent withheld

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A mass spectrometry-based approach for studying kinase activity

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Protein kinases play a key role in numerous cell signaling pathways. They have emerged as an important class of enzymes since aberrant kinase activity is associated with human cancers and inflammatory disorders. Whilst the human kinome is comprised of over 500 members, only a fraction have been exploited as drug targets for therapeutic interventions. Thus, many members of the kinase superfamily and their role in human diseases remain poorly understood. This can be attributed to the fact they are in low cellular abundance in complex protein samples and their detection is reliant on the quality of available phosphoantibodies. In recent years, efforts have been made to develop immobilized small-molecule kinase inhibitors as kinase-capturing reagents. When coupled with highly sensitive mass spectrometry analysis, this kinase enrichment step allows for the comprehensive scanning of the kinome and the identification of dysregulated kinase signaling networks in disease states[1].

In this study, we have used two kinase-capturing reagents to profile the kinome from various cellular sources including HEK293Ts, primary human neutrophils, murine natural killer (NK) cells and bone marrow-derived macrophages (BMDMs). Kinome enrichment was achieved using CTx-0294885 resin (KiNET-1 beads, SYNkinase) and a pan-JAK1/2 inhibitor CYT387 (Cytopia/Gilead) coupled to NHS-sepharose beads. In this way we identified a number of novel kinases within specific cell types, some of which may play important roles in the context of inflammatory diseases such as Gout and Rheumatoid arthritis. This kinome profiling strategy was performed alongside a phosphopeptide enrichment strategy for quantitative mass spectrometry analysis. Using this approach we identified a number of novel phosphopeptide sites within clinically-relevant kinases including the JAK protein tyrosine kinases. We identified 40 phosphorylation sites in murine JAK2 (23 novel and 17 known sites), whilst 43 phosphorylation sites were identified in murine JAK1 (32 novel and 11 known sites). Mutational studies are currently underway to screen a selection of evolutionarily-conserved candidate phosphorylation sites for functional relevance.

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Protein cleavage generates functional diversity on the surface of bacterial pathogens

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Bacterial pathogens are known to post-translationally cleave large mass, surface molecules during maturation but the extent to which processing occurs on the cell surface has largely remained unexplored. We have been characterising post-translational cleavage events in cell surface proteins produced by several pathogenic mycoplasma species. Cell shaving and cell surface biotinylation protocols in combination with LC-MS/MS and 2D-PAGE have been used to generate comprehensive datasets that identify the complement of cell surface proteins and predict cleavage sites, such as the now well-characterised S/T-X-F↓X-D/E motif found in the P97 and P102 adhesin families (1-7). Our complementary, orthogonal approaches show that adhesins, lipoproteins, non-classically secreted proteins, and proteins predicted by PSORTb to localise to the cytosol can be cleaved. To investigate the functional significance of protein processing, we have developed a series of affinity-capture protocols using heparin, fibronectin, plasminogen, actin and host epithelial cell surface antigens as bait (1-7). Cleavage fragments representing different regions of the same molecule have been shown to bind to different extracellular matrix components and host surface-associated molecules (1-7). Independent studies show that these interactions occur with physiologically relevant binding affinities suggesting that processing may be a mechanism to increase the repertoire of functionally diverse proteins on the cell surface of these genome-reduced pathogens.

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Proteome-Wide and Proteoform-Specific Host Cell Responses to Respiratory Syncytial Virus Infection

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Human respiratory syncytial virus (hRSV) is the most serious cause of lower respiratory tract infection in infants, young children and immunocompromised individuals. There are no licensed vaccines or efficacious therapeutics to combat hRSV. Innate immune responses are initiated upon host cell detection of viral-specific molecular patterns, such as virus-specific RNA

features. This detection system activates the production of immunomodulatory cytokines (induction), which in turn stimulate the production of antiviral protein effectors (signalling), some of which amplify the cytokine induction process. hRSV produces two small non-structural proteins (NS1 and NS2) which dampen the host cell innate immune system by targeting key players in both induction and signalling pathways. Therapeutic and vaccine developments to combat hRSV may emerge by gaining a better understanding of the interactions between hRSV NS1 and NS2 and host cell antiviral pathways. We have developed a comprehensive suite of proteomic approaches to identify specific targets of hRSV NS1 and NS2. This includes workflows based on in-solution protein IEF of cell lysates with downstream bottom-up mass spectrometric label free quantification of the differences between hRSV-infected and uninfected cells. Another workflow involves bottom-up analyses of digests of unfractionated whole cell lysates combined with label free quantification. Our infectious experiments include hRSV encoding the complete wild-type genome and genotypes lacking NS1 and/or NS2 coding capacities. As a consequence we have identified key pathways that are promoted and/or blocked in order to achieve survival of the infected host cell in a state conducive to hRSV propagation. Although some observations were common to both proteomic workflows, the in-solution IEF workflow uniquely enabled observation of some regulation at a global level due to selective enrichment of low abundance proteins. Furthermore, the in-solution IEF workflow provided information on regulation at a proteoform level that was not evident on a global quantification level. Of particular interest were the observations that NS1 appears to directly suppress the activation of NF-κB1 and to promote the survival of pro-apoptotic proteins within the infected cell. Furthermore, it was evident that hRSV has tactics to suppress the innate antiviral responses of the infected cell independently of NS1 and/or NS2. The strengths and weaknesses of these workflows will be discussed along with how specific observations provide a better understanding of how hRSV subverts host cell antiviral pathways.

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Secretion of Bioactive Compartment-Specific Neutrophil Proteins Displaying a New Type of Glycosylation in Pathogen-Infected Sputum

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Unlike plants and invertebrates, vertebrates reportedly lack proteins displaying truncated N-linked glycosylation (paucimannosylation) with the monosaccharide composition mannose$_{1-3}$fucose$_{0-1}N$-acetylglucosamine$_2$. Enabled by technology advancements in system-wide glycopeptide characterization (glycoproteomics), we document as the first that protein paucimannosylation is a significant host-derived molecularsignature in neutrophil-rich sputum from pathogen-infected human lungs and is negligible in pathogen-free sputum. Six paucimannose N-glycans were carried by inflammation-associated proteins of human neutrophil origin including myeloperoxidase, azurocidin and neutrophil elastase that localized specifically to azurophilic granules. Paucimannose *in vitro* synthesis by human azurophil-specific β-hexosaminidase A, together with promyelocyte stage-specific expression of paucimannosylated proteins and their biosynthetic enzymes, indicated a novel spatio-temporal biosynthetic route in early neutrophil maturation. Exogeneous generation of paucimannose was excluded by absence of bacterial exoglycosidase activity or paucimannosidic glycans. *P. aeruginosa* induced virulence-dependent secretion of paucimannosidic proteins from isolated and sputum neutrophils. Interestingly, paucimannosidic proteins displayed virulence-specific bacteriostatic effects towards *P. aeruginosa* and showed affinities to mannan-binding lectin suggesting multiple immune-related functions of paucimannosylation in activated neutrophils.

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The Metabolic Signalling Landscape

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Metabolic homeostasis is subject to major challenges particularly those imposed by starvation, diet and exercise. There has been considerable interest in dissecting the adaptations that occur in response to these kinds of perturbations and which of these are most likely to contribute to defects or constitute major points of regulation. As one approach toward addressing this challenge we have embarked upon global analysis of protein phosphorylation networks in either adipose tissue or skeletal muscle under different perturbation states including insulin stimulation, exercise or insulin resistance. This has unveiled the complex nature of these perturbations as well as a surprising heterogeneity in terms of the biological pathways that participate in metabolic homeostasis.

The details of some of these networks will be presented together with specific examples of some of the novel features that have merged from this kind of research.

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Revealing proteomic and metabolomic changes associated with lens cataract formation with Imaging Mass Spectrometry

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The lens is a unique structure in the eye that focusses light onto the retina in order to form a sharp image, and has several adaptations to do this effectively. It lacks a blood supply, has an ordered cellular structure, and loses light scattering cellular organelles as lens fibre cells mature. Since it is a large avascular tissue, the lens requires a unique transport system that delivers nutrients and removes waste products from the lens centre. Breakdown of this system is thought to lead to lens protein modification and cataract formation, the leading cause of blindness worldwide. Cataract is characterised by opacities that form in distinct regions of the lens, and results from both genetic and environmental factors. Furthermore, biochemical studies into the aetiology of lens cataract have shown that metabolic changes associated with oxidative damage precede the proteomic changes that lead to cataract formation.

MALDI imaging mass spectrometry was utilised to map metabolomic and proteomic changes that lead to cataract formation in a laboratory model of age-related cataract. Bovine lenses were removed from the ocular globe and subjected to hyperbaric O_2 treatment to simulate age-related cataract. Lens cryosections were collected on MALDI targets, and matrix (9-aminoacridine or sinapinic acid) applied. MALDI imaging data sets were collected at 150-250mm spatial resolution using a MALDI-FTICR mass spectrometer (for metabolites) or a MALDI-TOF mass spectrometer (for proteins).

Hundreds of signals for small molecules were detected, including major lens antioxidants, nucleotides and purinergic signalling molecules. For proteomic studies, unmodified and modified forms of the abundant lens protein alpha crystallin were mapped. The effect of hyperbaric oxygen treatment on metabolite and protein distributions will be discussed. This study highlights the complex aetiology of lens cataract and may suggest novel targets for anti-cataract therapies.

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Translation of Membrane Proteome Interactions into Novel Colorectal Cancer Targets

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Our past works have shown that that integrin (avb6) and protease receptor (uPAR) expression are upregulated in colorectal cancer (CRC), allowing us to understand changes associated with the epithelial metastatic phenotype. Here, we show how immunoprecipitation allows identification of uPAR- and avb6-interacting membrane proteins (i.e., *metastasome*) and downstream signaling events are linked. We also demonstrate how the metastasome is involved in regulation of cellular invasive characteristics, including the switch of TGFb1 from a suppressive to an aggressive factor in late stage colorectal cancer. Analyses of the precise sites of protein interaction between uPAR and integrin avb6 using a plethora of methods, including peptide arrays, peptide competition assays, *in silico* modelling, inhibitory peptide (iPEP) studies and proximity ligation confirm the interaction and suggest interesting biologies that have implications for development of new therapies targeted at this interaction. These studies have developed lead iPEPs and are now focussed on small molecule compounds that antagonise this particular metastasome. Both lynchpin proteins (uPAR and avb6) are independently shown to be poor epithelial prognostic indicators, especially in Stage B rectal cancer. Despite this data, little is known regarding patient outcome when both factors are co-expressed. We propose a new model for how signal transduction "switches" require pre-formation of a precise membrane interactome composition. In addition, our recent rectal cancer study demonstrates that uPAR is expressed both in the stromal and/or epithelial compartments (presumably with different interactomes since avb6 is epithelially-restricted) and that high expression in these different compartments results in opposite clinical survival outcomes (high epithelial rectal cancer uPAR levels poorer prognosis; high stromal expression better prognosis). We are exploring if the ratio of these uPAR measurements provides a better overall clinical prognostic indicator. Collectively, our data demonstrate combination(s) of clinical proteomics and cell biological methods result in deep, comprehensive membrane proteome understanding in cancer, providing a potential new CRC therapeutic target.

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Proteomic profiling of an inducible model of acute myeloid leukaemia reveals novel insights into leukaemogenesis.

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The Mixed Lineage Leukemia (MLL) gene located on chromosome 11q23 is a frequent site of translocation that results in a number of oncogenic fusion proteins. MLL-translocations are present in a variety of paediatric and adult leukaemias and are associated with poor survival outcome. Despite intense research efforts the mechanisms underlying the leukaemogenic activity of MLL-translocations are poorly understood. Our aim was to identify proteins involved in leukaemogenesis that are regulated by MLL-translocation gene expression through quantitative proteome analysis using a leukaemia model. Our model of acute myeloid leukaemia (AML) is controlled by the regulated expression of a common MLL-translocation gene, MLL-AF9. Initially, surface captured proteins were analysed over a time course using a SILAC quantitative proteomics workflow and high-resolution mass spectrometry to generate a profile of proteins that are expressed in the presence or absence of MLL-AF9 expression. We extended this analysis by also profiling changes to the entire leukaemia proteome during MLL-AF9 regulation. The results from these two techniques have not only confirmed several known targets in AML but highlighted novel pathways and targets that are upregulated when MLL-AF9 is expressed. These pathways include epigenetic regulators, cell signalling and cell-cell or cell-extracellular matrix interactions. The results from this study not only identify mechanisms by which MLL-AF9 regulates leukaemogenesis, but also demonstrate the potential identification of biomarkers and novel drug targets.

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Functional and biochemical analysis of HLA ligands reveals the molecular basis of HLA associated adverse drug reactions.

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Adverse drug reactions (ADRs) cost the Australian health care system over $660 million annually and complicate the use and public trust of key pharmaceuticals. In recent years numerous associations between immune mediated ADRs and specific Human Leukocyte Antigen (HLA) alleles have been identified. These molecules are cell surface glycoproteins responsible for the presentation of peptides, derived from the breakdown of both endogenous and exogenous proteins, at the cell surface for immune surveillance. In health, constitutive HLA-peptide complexes are inert, whilst HLA molecules in complex with novel or foreign peptides (e.g. those derived from invading pathogens) may stimulate a T cell response. We recently defined the mode of interaction of the anti-retroviral drug abacavir with HLA-B*57:01, the allele associated with the potentially lethal ADR, abacavir hypersensitivity syndrome. Using a combination of mass spectrometry and structural biology, we showed that abacavir bound within the peptide-binding groove of HLA-B*57:01 altering the selection of self-peptides, causing significant perturbation of the immunopeptidome (the peptides displayed by the HLA), and presenting T cells with countless novel, immunogenic, HLA-B*57:01-abacavir-self-peptide complexes[1]. Here, we extend these studies to other ADRs and use mass spectrometry (targeted and data dependent acquisition methods) and T cell activation assays to monitor drug induced changes in the immunopeptidome and the immunogenicity of ADR associated HLA molecules. Recent data on the application of these techniques to investigate interactions between allopurinol (and its metabolites) and HLA-B*58:01, implicated in allopurinol hypersensitivities such as the severe cutaneous reactions Stevens-Johnson syndrome and Toxic Epidermal Necrolysis, will be discussed.

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Tales from protein TAILS: the protease web deciphers the N terminome

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Biological systems are complex and so model systems leading to models to predict human biological processes are required for mechanistic understanding of physiological pathways in all biological events and responses, signaling pathways and their perturbations in disease, and drug target validation. For example, interactions of proteases and inhibitors through protein cleavage and inhibition play crucial roles in the regulation of biological systems. Well studied as classically described protein cascades *e.g.* the complement system and coagulation, or newer recognized cascades of kallikreins in skin and caspases in apoptosis, these interactions have been proposed 10 years ago to expand pervasively in a network termed the protease web.

We compiled large-scale data of protease web interaction and expression to investigate the regulatory potential of the protease web. Using graph theory to represent the protease web of annotated cleavage and inhibition information, we investigated connectivity in the protease web. We identified a pervasive human protease web, where interactions between protease classes and cascades are common with protease inhibitors, in particular serpins, often forming network hubs. The model describes how proteases can influence the cleavage of many more proteins than their direct substrates through protease activation pathways or inactivation of protease inhibitors. Our model successfully predicted a perplexing proteolytic pathway *in vivo,* where activities of MMP8 and neutrophil elastase are linked by an inactivating cleavage of serpin A1 by MMP8 that we experimentally validated. We also found that protein interaction databases frequently contain protease interactions, in particular inhibitions, and improved our model of the protease web. Degradomics analysis using TAILS N-terminomics allowed us to dissect proteolysis in skin inflammation and demonstrated the interconnectivity of the protease web and new roles for proteases in vivo. By removing serpin blocks, MMPs flip the "Metallic Serpin Switch" and so dynamically regulate serine protease cascades by altering the balance between intact inhibitory serpins and inactivated cleaved serpins.

Our findings supply systematically derived and validated evidence for the existence of the protease web, where interactions are potentially broad but are fine tuned by expression and localization of interactors leading to specific interactions in- and outside cells and in specific tissues.

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Characterisation of the peptide and protein content of ant venoms for use as bioinsecticide and antimicrobial leads

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Animal venom peptides are currently being developed as novel drugs and bioinsecticides. Given that ant venoms are used for predation and defence, their venom is a potential source of these peptides. Also, venomous ants are an untapped source of these lead compounds as they remain extremely understudied. Initially, the protein/peptide components of seven different ant species were characterised. 1D and 2D gels of the venoms revealed proteins ranging from <10 kDa to >250 kDa. NanoESI-QTOF-MS/MS analysis confirmed the presence of common venom proteins and many undescribed proteins. C18 reverse-phase (RP) HPLC separation followed by LC-MALDI-TOF MS of the venoms was then undertaken, this revealed considerable heterogeneity in the HPLC and mass profiles of the five venoms. After optimisation of the MALDI matrices, it was found that venoms contained between 144–1032 peptides. Most of these peptides lie between 1–5 kDa. Disulfide-bonded peptides were also identified in all ant venoms (range 2–28) using the reducing MALDI matrix, 1,5-DAN. Insecticidal activity of whole venom was determined using

house crickets. Of the four venoms tested thus far, three venoms showed significant paralytic and lethal activity. Bioassay-guided fractionation using cation exchange and RP-HPLC of three venomsis currently being undertaken to identify insecticidal neurotoxins. The antimicrobial activity of venoms and toxins is also presently being investigated using MIC (minimum inhibitory concentration) assays with both gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli*) to identify potential anti-microbial agents. In conclusion, these venoms represent a vast and untapped source of potentially novel bioactive drug and insecticide leads.

A novel, ultrasensitive approach for quantitative carbohydrate composition and linkage analysis using nanoLC-ESI ion trap tandem mass spectrometry

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Abstract

Introduction

Glycosylation is one of the most complex yet common post translational modifications, which drastically enhances the functional diversity of proteins and influences their biological activity. Identification and characterisation of glycans is an important step in correlating glycan structure to its biological function. Structural assignment of glycans (glycomics) and their compositions based on MS analyses is often based on taking well studied glycosylation pathways for granted. Nevertheless, many monosaccharide building blocks are indistinguishable by mass alone and detailed linkage information is also not easily obtained by MS/MS analyses. In particular when studying glycosylation from less well studied organisms such as prokaryotes, monosaccharide analysis is indispensable to profile the composition of complex carbohydrates present in glycoconjugates.

Methods

Carefully designed sequential permethylation and reductive amination steps prior and after acid hydrolysis enable separation and differentiation of the various monosaccharides and their respective linkage positions. Separation of various derivatised monosaccharides was achieved using Reversed Phase – Liquid Chromatography (RP-LC)-ESI-MS/MS. In addition, absolute quantitation is accomplished including a set of internal standards, thus providing qualitative and quantitative information on the monosaccharide residues present in a specific sample.

Results

Various derivatised monosaccharides alditols were identified based upon their retention time along with mass spectrometric detection. The resulting fragment ions observed in the MS/MS spectra arise from the cleavage of methyl groups are diagnostic for the each substitution pattern. These differences allowed establishment of 'fragmentation pattern' for these derivatised monosaccharide alitols. Furthermore, we have established fragmentation pattern library for various biologically important N-glycans for automated linkage analysis.

Conclusion

The method reported herein has a limit of detection of ≤ 250 fmol for all the monosaccharide analysed and is sensitive to as low as 12 pmol of initial analyte, which now allows to perform both, glycomics and monosaccharide analyses from low µg amounts of initial glycoprotein. The present method reported here does provide more confidence in correlating the structure and stereochemistry of monosaccharides present in any glycoconjugates, thereby increasing the confidence in glycan structure assignments obtained from glycomics experiments.

Novel Aspect

The methodology reported describes for the first time, a simple and sensitive method using Reversed Phase – Liquid Chromatography (RP-LC)-ESI-MS/MS for unambiguous identification and linkage determination of various monosaccharides (including N-acetylneuraminic acids).

EVALUATING THE RELATIONSHIP BETWEEN *N*-GLYCOSYLATION AND PROTEIN STABILITY IN *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. *C. jejuni* was the first prokaryotic organism found to possess an *N*-linked protein glycosylation system. Approximately 100 *C. jejuni* proteins to date have been shown to be targets of this post-translational modification[1,2], and this system has been shown to be a crucial for pathogenicity albeit through an as yet undefined mechanism. Here, 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* strain 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[3]) was also employed for pair wise comparisons of the *N*-degradome of wild-type JHH1 and individual *pgl* knock out strains to address the hypothesis that the addition of the *N*-linked glycan may provide protection from proteolytic degradation for the largely unstructured regions of the protein on which they're commonly attached. 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.

These proteomics-based approaches were complemented with various standard phenotypic tests to establish if the observed effects of loss of *N*-glycosylation extended to broader changes in *C. jejuni*'s physiology.

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TGFβ-1 increases cell proliferation, wound healing and induces changes to membrane proteome in colorectal cancer cells with varying integrin β6 expression

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It is well understood that Transforming Growth Factor-β (TGFβ) signalling is a potent regulator of cell growth, differentiation, migration and tumour suppression. However, during colorectal cancer (CRC) and other cancers TGFβ mRNA and protein show marked increases are associated with tumour growth. The involvement of TGFβ as tumour promoter is poorly understood. We hypothesise that the increased expression of TGFβ can be achieved by high expression levels of integrin αvβ6, which can activate latent-TGFβ and MMP-9 both of which are activators of latent-TGFβ. High expression of integrin αvβ6 has been observed in various cancers including CRC. Cell proliferation and would healing assays showed significant differences when treated with active TGFβ. To gain more in-depth understanding of role of TGFβ, αvβ6 and uPAR in CRC we compared the membrane proteome of TGFβ1 (10ng/mL) stimulated SW480 cells with none or overexpressing integrin β6 using iTRAQ. We identified 2666 proteins, out of which 510 proteins were from the plasma membrane. The IPA analysis with significantly altered proteins revealed c**ellular assembly and organization, cell-to-cell signalling and interaction as the top-ranked altered biological function. Ingenuity pathway analysis also showed** RAN and EIF2 signalling were amongst top 5 canonical pathways identified. **We identified various proteins including integrins (α3β1, α4β1, α6β1, α5β1, αv), ERK1/2, TGFβ and MAPK1/2 through IPA network analysis. All these molecules have been previously implicated in various cancers including CRC. The results show that TGFβ signalling is enhancing the ability of the cancer cells towards progression and metastasis. Therefore, it is very important to study the TGF**β associated protein-protein interactions during CRC which will serve to identify novel key players for metastasis, and serve as biomarkers and/or drug targets to improve CRC therapy.

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Comprehensive characterization of phosphorylation sites of Newcastle disease virus proteins by a multitude of fragmentation techniques

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Paramyxoviruses are a family of negative stranded RNA viruses whose members comprise of serious human pathogens, such as measles virus, mumps virus and respiratory syncytial virus; and in animals, such as Newcastle disease virus (NDV) and rinderpest virus. Apart from its potential economic impact on the poultry industry worldwide, NDV is becoming a promising agent for oncolytic virotherapy of mammalian cancers.

For productive infection viruses must hijack the host cellular machinery as they cannot self-reproduce. To avoid elimination they have to weaken host antiviral responses but at the same time utilize the host intracellular trafficking and biosynthetic systems to enter and exit infected cells while producing new viral particles. These variegated interactions expose viral proteins to numerous host signaling pathways that might post-translationally modify them leading to a change in their functions either to favour host cell preservation or proliferation of infection. In either case these post-translational modifications (PTMs) on viral proteins serve as markers that reflect viral-host protein-protein interactions and provide a useful means of identifying them, regardless of the functional implications of these modifications. In addition, cellular localization of these interacting host proteins indicates intracellular trafficking behaviour of the viral proteins during the infectious cycle.

In this pursuit, we have extensively characterized phosphorylation sites of the avian paramyxovirus-1, NDV, proteins by using an array of fragmentation techniques on several advanced mass spectrometry (MS) platforms. This includes analysis of unfractionated and phosphopeptide enriched digests from lysates of purified virions by ion trap (IT) CID with multi-stage activation, IT-ETD, IT-ETciD, IT-EThcD, Orbitrap (OT)-HCD and IT-HCD. These multitudes of fragmentation techniques on different MS platforms have provided common and unique phosphopeptide identifications in NDV proteins. Importantly, diverse and distinctive peptide backbone cleavage favoured by different fragmentation techniques has enabled unequivocal assignment of phosphosites detected in NDV proteins.

Quantitative proteomic analysis of Cabernet Sauvignon grape cells exposed to thermal stresses reveal alterations in sugar and phenylpropanoid metabolism

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Grapes (*Vitis vinifera*) are a valuable fruit crop and wine production is a major industry. Global warming and expanded range of cultivation will expose grapes to more temperature stresses in future. We investigated quantitative shotgun proteomic changes induced by temperature stresses on Cabernet Sauvignon cells to provide insights into metabolic pathways related to temperature stress in grapevines. Our study examined proteomic changes induced by the impact of four different temperature stress regimes, including both hot and cold temperatures, on cultured grape cells. Cabernet Sauvignon grape cell suspension cultures grown at 26°C were subjected to 14 hours of exposure to 34°C and 42°C for heat stress, and 18°C and 10°C for cold stress. Cells from the five temperatures were harvested in biological triplicates. Proteins were extracted using methanol-chloroform [1] and digested by filter aided sample preparation (FASP) protocol [2]. Proteins were identified with a Velos-Pro linear ion-trap mass spectrometer by gas phase fractionation (GPF) [3], and spectra were searched against UniProtKB *Vitis vinifera* database and quantified by spectral counting. A total of 1755 non-redundant proteins were identified from the five temperature points. Ninety-eight proteins were identified exclusively in extreme heat stress (42°C) and ninety-five proteins were identified exclusively at extreme cold stress (10°C). Gene Ontology (GO) annotations of differentially expressed proteins provided insights into the metabolic pathways that are involved in temperature stress responses in grape cells. Sugar metabolism displayed switching between alternative and classical pathways during extreme temperature stresses. Nine proteins involved in the phenylpropanoid pathway were highly up-regulated at extreme cold stress, and numerous other proteins involved in phenylpropanoid metabolism and anthocyanin synthesis were found to be cold-responsive proteins. This is the first label-free shotgun proteomic study on grapes exposed to hot and cold temperature stress.

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Understanding ovine skin properties using MALDI IMS and nano-LCMS

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New Zealand produces large quantities of sheep pelts, which are largely used to make garment leather. Leather from conventionally processed sheep skins is not strong enough for footwear. Therefore, converting them to footwear leather would not only increase their raw material value but also be of more benefit to the NZ sheep leather industry as it targets the more stable footwear market rather than that of fashion-dependent clothing.

Although much research has been carried out into the physical structure and arrangement of collagen fibers/fibrils of leather and the effects of this on strength, the chemical characteristics and molecular composition of leather is less well understood.

It is known that raw sheep skins are strong but that their strength is lost in the process of leather making, which removes unwanted skin protein components in order to achieve desired properties, especially softness and flexibility in the leather. Using a proteomics approach would allow us to identify structural proteins in raw sheep skin. Comparing proteins from raw sheep skin to pickled sheep skin will give us information on what proteins have been lost during the leather process.

Here, we describe a survey of the identified skin proteome in raw and pickled sheep skins using a gel approach based on chip based nano-LC-MS/MS (Agilent Q-TOF 6520) and MALDI IMS (Autoflex III, Bruker). In the former method, solubilised skin proteins were separated by conventional 1D-gel electrophoresis prior to trypsin digestion. Trypsin digested peptides were then separated by nano-LC and identified using TPP (trans-proteomics pipeline), searching against the mammalian NCBI database. Protein matches were retained less than 5% FDR with at least two different unique peptides. The second method was an on-tissue approach applying trypsin directly on to the tissue sections. This is a powerful technique for mapping biological molecules such as endogenous proteins and peptides as it detects molecular species present and visualizes their distributions in a single tissue section. The two techniques combined provide us with high confidence in any identified proteins.

LC-MS timescale intact proteoform profiling

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All along their life cycle, proteins undergo various transformations that can alter their functions while keeping a good part of their primary sequence intact. These multiplication of PTM patterns, alternative splicing forms or products of proteolitic processing

cannot be simply resolved with a bottom-up approach, as very few peptides are specific from the given proteoform. However, the information relative to the distribution of different proteoforms is encoded in their intact masse. Being able to catch this information efficiently will drive the biologist into a new analytical dimension, far beyond the traditional gel-based approaches.

Bruker has an history with the analysis of intact proteins, whatever it is with NMR, X-ray Crystallography, Infrared or MS, accessing all structural information, from the primary to the quaternary structure. Bruker's UHR ESI-Q-TOF systems are now capable of delivering accurate mass and isotopic pattern information for hundreds of protein in a mixture within minutes. In the exemple we present, the combination of dynamic range, broadband transmission and sensitvity makes it possible to resolve several hundreds of proteins from a E.Coli and yeast Lysates in less than 20 minutes. The Dissect™ and SNAP™ algorithms enable to extract the information of co-eluting proteins and retrieve the monoisotopic mass from any isotopically resolved protein, therefore enabling to reveal the proteoforms distribution patterns. First insights in the quantitative capabilities of the approach will be illustrated.

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N-terminal Protein Sequences Highlight the Extent of Proteolytic Processing in Pathogenic Bacteria

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Proteolytic cleavage is a ubiquitous post-translational modification of proteins, responsible for protein signalling, activation, localisation and ultimately degradation. Due to a variety of experimental limitations this important physiological process has been largely understudied, particularly in prokaryotes and archaea. Proteases and proteolytic processing have been linked to virulence in a variety of infectious diseases. Therefore experimental investigation of proteolytic cleavage is needed to determine the extent and functional roles of protein processing in the production of mature proteins.

The primary focus of this study is to sequence the N-terminal sequences of mature protein products of prokaryotic pathogens, and to characterise the resulting mature protein fragments. N-terminal sequence data can be used to identify true protein start sites and any downstream post-translational processing. The genome-reduced agriculturally-important pathogen *Mycoplasma hyopneumoniae,* was selected as a model organism for these studies. There is a large body of literature demonstrating extensive proteolytic processing of many highly expressed adhesins which are critical for pathogenesis.

Using dimethyl labelling of protein amine groups, including native N-termini, the data obtained from mass spectrometry analysis has provided evidence confirming the start sites of protein translation and complementary data pinpointing the exact sites of proteolytic cleavage. In addition, the data indicates that an unprecedented number of cytosolic proteins are targets for post-translational processing on the cell surface of pathogenic bacteria. Many of these proteins have been described as "moonlighting" proteins in the literature and have been implicated in pathogenesis in other organisms.

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Hydrolysis of maleimide–peptide adducts reduces sensitivity when interrogating proteomes for thiol oxidation

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Oxidative stress, caused by reactive oxygen and nitrogen species (RONS), is widely recognized as important in both the pathogenesis and subsequent pathophysiological sequelae of many diseases, including diabetes, cancer, and muscular dystrophy. A key target of RONS is the thiol group of protein cysteine residues. As thiol oxidation can affect protein function, mechanistic information about how oxidative stress affects tissue function can be ascertained by identifying oxidized proteins.

The most popular proteomics-based method for interrogating protein cysteine oxidation is via the alkylation of thiols using iodoacetamide chemistry, as in the OxICAT adaptation of the ICAT kit. However, this method is suboptimal for assessing the labile oxidative modification of cysteine residues as iodoacetamide chemistry has been shown to result in incomplete, non-specific, and slow alkylation of protein thiols.

Alkylation by maleimide chemistry is complete, highly selective, and rapid. It is therefore preferred to iodoacetamide. However, we find that in a standard proteomic workflow, significant hydrolysis of the maleimide adducts (succinimides) occurs with associated +16 m/z and +18 m/z peaks in the mass spectrum.

Indeed, when losses during a protein sample preparation workflow were tracked using 1D gel electrophoresis and MALDI-TOF mass spectrometry, it was found that up to 89% of the alkylated peptide can be hydrolysed during sample preparation. Gel electrophoresis alone resulted in an average 6.4% ± 3.9% (n = 3, mean ± SEM) hydrolysis; coomassie staining of gels 25% ± 6.8%, tryptic digests of gel bands 17% ± 21%; extraction of peptides from gel bands 13% ± 2%; and biotin/streptavidin purification 30% ± 2%. The cumulative loss of signal for the peptide adduct at the expected m/z is so large that it can it to fall under the limit of detection, leading to incomplete profiling of the redoxome. Strategies to minimize succinimide hydrolysis will be presented.

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The Analysis of bead-based multiplex immuno-assays for accurate discovery of cytokine, chemokines, and growth factors expression within plasma

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Small proteins found in plasma such as cytokines, chemokines and growth factors are involved in aging, disease pathogenesis, embryonic development, non-specific response to infection, specific response to antigens, and changes in cognitive functions. In addition, such analytes have roles in stem cell differentiation, vaccine efficacy and allograft rejection. Often the expression of these analytes are obtained from bead based multiplex immunoassays such as Luminex's xMAP® technology. Software systems such as xPONENT® from Luminex and Bio-Plex® Manager™ output tabulated sheets containing observed concentration values, fluorescent values and standards and expected concentration values as well as background corrected values for test, controls, standards and blanks samples. However, from such systems much of the life scientist's test data can end up classified as being out-of-range leaving him or her with the problem of working with sparse and unbalanced data that in turn reduces their power of discovery and analysis. We have developed new approaches to the analysis of bead-based immunoassays that can reduce the number of out-of-range values by 50%. We show that basing the analysis on raw and mean fluorescent values is often a better choice than relying on the derived concentrations values obtained from commercial software systems. We show how to analyse and to normalize the test data to compensate for plate-to-plate variations, how to check the reliability of test samples even if all or most of the concentration data is deemed out-of-range, and we show how to get improved estimates of analyte concentrations. In this study we use the expression of 14 analytes, using 6 commercially available kits, across 177 patients, recorded at two time points and the associated analyte standards, controls and blanks. In total 60 micro titre plates are used resulting in 4965 readings. We show that the common practice of subtracting analyte blanks from test samples has no rational basis. We argue for our analysis of protein expression that the fluorescent values are a better choice than absolute concentration values. The significance of this work for the life scientist means higher statistical power and even lowers experimental costs.

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MALDI imaging mass spectrometry of N-linked glycans on formalin-fixed paraffin-embedded murine kidney

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Recent developments in spatial proteomics have paved the way for retrospective in situ mass spectrometry (MS) analyses of formalin-fixed paraffin-embedded clinical tissue samples. This type of analysis is commonly referred to as matrix-assisted laser desorption/ionization (MALDI) imaging. Recently, formalin-fixed paraffin-embedded MALDI imaging analyses were augmented to allow in situ analyses of tissue-specific N-glycosylation profiles. In the present study, we outline an improved automated sample preparation method for N-glycan MALDI imaging, which uses in situ PNGase F-mediated release and measurement of N-linked glycans from sections of formalin-fixed murine kidney. The sum of the presented data indicated that N-glycans can be cleaved from proteins within formalin-fixed tissue and characterized using three strategies: (i) extraction and composition analysis through on-target MALDI MS and liquid chromatography coupled to electrospray ionization ion trap MS; (ii) MALDI profiling, where N-glycans are released and measured from large droplet arrays in situ; and (iii) MALDI imaging, which maps the tissue specificity of N-glycans at a higher resolution. Thus, we present a complete, straightforward method that combines MALDI imaging and characterization of tissue-specific N-glycans and complements existing strategies.

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Improvements in 2D gel electrophoresis for biomarker discovery in plasma proteins

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Publish consent withheld

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A Label-Free Multi-Omic Study of a Glucosylceramide Inhibitor Effects on Obesity

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Obesity is one of the risk-factors associated with metabolic syndrome, causing excess body fat to be accumulated to the extent that it adversely affects health and life expectancy. It has previously been demonstrated that glucosylceramides play a crucial part in such metabolic syndromes. The manipulation of the function of glucosylceramides with small molecule drug compounds within mouse models has shown that symptoms can be negated. Knowledge relating to the proteome, metabolome and lipidome during development is still to be fully explored. The work presented here is to provide a multi-omic analysis of protein and lipid liver extracts from control and obese mouse models undergoing treatment to prevent or revert obesity.

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Improved HDX Workflow for On-line Digestion, Separation and Data Analysis

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Hydrogen Deuterium Exchange (HDX) experiments on proteins can reveal detailed information about protein tertiary structure (folding/packing) and quaternary structure (protein-protein interactions). Several key components of a Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS) workflow include reproducible sample preparation, on-line digestion, chilled chromatography, MS and dedicated informatics tools. Each of these components have been improved in recent years and in this study we demonstrate optimizations in microscale chromatography that improve both pepsin digestion efficiency and the resolution of the reverse phase peptide separation. The practical results are improved protein sequence coverage and more reproducible HDX experiments.

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Overexpression of αvβ6 integrin alters the colorectal cancer cell proteome in favour of elevated proliferation and a switching in cellular adhesion which increases invasion

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Many proteins enhance cancer progression towards life-threatening metastases. These include linking proteins called integrins which mediate cell adhesion to the extracellular matrix (ECM), consequently altering both function and phenotype. Specific neoexpression of the β6 integrin subunit correlates with the epithelial-to-mesenchymal transition, metastasis and with poor overall patient survival. Whilst b6 is implicated in these processes, exactly how it affects signaling and/or proteolytic pathways in metastasis remains unclear. A membrane-enriched peptide IPG-IEF shotgun proteomics study was undertaken comparing subclones of the SW480 CRC cell line transfected with a vector inducing unregulated β6 integrin overexpression against the 'empty' mock vector control cell line. β6 overexpression induced a significant change in 708 proteins found localised across most intracellular locations, some involving cellular processes and pathways underpinning cancer progression. β6 expression increased cell proliferation 4-fold, while decreasing cell adhesion to many integrin-ECM substrates. β6 expression also enhanced cell invasion and promoted the expression/repression of many established cancer-related pathways.

Characterisation of Monoclonal Antibody Drug Candidates and Biosimilars Using Mass Spectrometry

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The selective and specific recognition properties of antibodies are fundamental to many applications in biomedical research. The use of monoclonal antibodies (mAbs) as therapeutic drugs has proved to be highly successful, and as patent rights run out, an increasing number of biosimilar antibodies are being developed.

The structural characterisation of mAbs is one essential requirement for their commercialisation. Mass spectrometry (MS) is a proven analytical tool for proteomics and allows the sensitive and high resolution characterisation of proteins, including mAbs.

In our work, we have applied an array of MS techniques to characterise several commercially available therapeutic mAbs. To first achieve baseline resolution of an IgG mAb, we performed intact protein mass measurement on a high resolution mass spectrometer. The relative abundance of glycans present on the antibody heavy chain was then determined using a mAb-Glyco chip LC-MS system (Agilent Technologies). To characterise the primary structure the antibody was reduced and alkylated then digested using three different enzymes (Trypsin, Glu C, Lys C) and microwave assisted acid hydrolysis. The acquired peptide MS/MS data was matched against the predicted peptide fragments for the antibody heavy and light chains. The combined results from each enzyme digestion achieved close to 100% sequence coverage for both the heavy and light chains. Disulfide bridges were determined by digestion with trypsin or GluC/Trypsin, followed by LC ESI MS/MS. A theoretical disulfide bond peak list was generated using in-house software and MasterView (AB Sciex) was used to screen the LC ESI MS/MS data and identify peptides with disulfide bonds.

This workflow demonstrates how MS can be used to generate accurate and comprehensive structural information for biomedically relevant mAbs.

Isolation and identification of *Enterococcus faecalis* membrane proteins using membrane shaving and one-dimensional SDS-PAGE coupled with mass spectrometry.

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Background: *Enterococcus faecalis* is a significant nosocomial pathogen which is able to survive in diverse environments and resist killing with antimicrobial therapies. The expression of cell membrane proteins play an important role in how bacteria respond to environmental stress. As such, the capacity to identify and study membrane protein expression is critical to our understanding of how specific proteins influence bacterial survival. Here we describe a combined approach to identify membrane proteins of *E. faecalis* ATCC V583 using membranes fractionated by either 1D-SDS-PAGE or membrane shaving, coupled with LC-ESI mass spectrometry.

Results: Two hundred and two membrane-associated proteins were identified which represents approximately 24 percent of the predicted membrane-associated proteome. 170 were isolated using 1D-SDS-PAGE and 68 with membrane shaving, with 36 proteins being common to both techniques. Ninety seven percent of the proteins identified by membrane shaving were membrane associated with the majority being integral membrane proteins (89%). The majority of proteins identified with known physiology are involved with transportation across the membrane.

Conclusion: The combined 1D-SDS-PAGE and membrane shaving approach has produced the greatest number of membrane proteins identified from *E. faecalis* to date.

Characterisation of the interaction of heterodimeric αvβ6 integrin with urokinase plasminogen activator receptor (uPAR)

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Urokinase plasminogen activator receptor (uPAR) and the epithelial integrin αvβ6 play critical roles in cancer metastasis. Importantly, both these interacting molecules have been implicated in epithelial-mesenchymal transition (EMT) changes that facilitate escape of metastatic cells from tissue barriers. This study aims to characterise the uPAR•αvβ6 in ovarian and colon cancer cells.

The study uses orthogonal in cellulo and in vitro approaches to characterise the uPAR•αvβ6 interaction on OVCA429 (ovarian cancer cell line) and four different colon cancer cell lines with modified β6 subunit expression (SW480Mock, SW480β6OE, HT29Mock and HT29β6AS). Proximity ligation assays (PLA) was used to confirm the previously identified uPAR•αvβ6 interaction

site. Peptide arrays were used to identify the specific sites of interaction which also validates our PLA studies. In silico structural analysis was then used to identify the most likely uPAR•αvβ6 interaction sites based on the peptide array data.

PLA study confirmed the uPAR•αvβ6 interaction in our cell lines. PLA studies were further validated using peptide arrays to identify potential physical sites of uPAR•αvβ6 interaction and as well as interactions with other known uPAR partners (e.g., uPA and vitronectin) and individual integrin subunits (i.e., αv, β1, β3 and β6). Our data suggests that interaction with uPAR requires expression of the complete αβ heterodimer rather than either individual subunits (i.e., αv, β1, β3 or β6). Finally, from the in silico structural analysis, it appears the most likely unique site/s of interaction of αvβ6 with uPAR are located in uPAR domain II and domain III. Functional consequences of disrupting uPAR•αvβ6 as a novel therapeutic target to ablate metastasis are explored. (Pubmed ID: 25318615)

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There's a hole in my assay, dear ELISA, dear ELISA: Using MS to detect hydrolysed gluten in beer that is responsible for false negatives by ELISA

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Gluten is the collective name for a class of proteins found in wheat, rye, barley and oats. Eating gluten triggers an inappropriate auto-immune reaction in ~70 million people globally affected by coeliac disease, where the gut reacts to gluten proteins and this triggers an immune response, resulting in intestinal inflammation and damage. Gluten-free foods are now commonplace, however, it is difficult to accurately determine the gluten content of products claiming to be gluten-free using current methodologies as the antibodies are non-specific, show cross-reactivity and have different affinities for the different classes of gluten. The measurement of gluten in processed products is further confounded by modifications to the proteins that occur during processing and in some case hydrolysis of the proteins. In this study, LC-MS/MS analysis of barley-derived beers revealed that certain classes of hordein were prone to hydrolysis (B- and D-hordein). Strikingly, those beers that contained high levels of B-hordein fragments gave near zero values by ELISA. The hydrolysed fragments that persist in beer show a dose-dependent suppression of ELISA measurement of gluten despite using a hordein standard for calibration of the assay. Using 2D gels the complete suite of hordeins present in barley grain, the precursor to malt using in brewing, were visualised. Further, using a combination of high resolution discovery proteomics and targeted quantitative proteomics the "gluteome" was characterised facilitating selection of peptide markers specific to each hordein family. The development of MS-based methodology for absolute quantification of gluten is required for the accurate assessment of gluten, including hydrolysed forms, in food and beverages to support the industry, legislation and to protect consumers suffering from coeliac disease.

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Characterization of an improved Ultra High Resolution Quadrupole Time of Flight (UHR-Q-Tof) Instrument for Proteomic applications.

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In shotgun proteomics it is desirable to identify and quantify a large number of individual peptides from complex samples, such as tryptic digests of human plasma samples or whole cell lysates in the shortest possible time. Complexity and concentration range, however, pose a great challenge to the MS instrumentation in terms of sensitivity, resolution and dynamic range. Several hardware modifications of a bench-top UHR-TOF instrument were carried out and evaluated addressing these particular performance aspects.

To test the impact of these modifications on proteomics performance, different complex tryptic digests (Lysates of Escherichia coli, Saccharomyces cerevisiae, human plasma) were mixed with stable isotope labeled peptides or digests of standard proteins at known concentrations, spanning a range of several orders of magnitude. Samples were analyzed with nano-flow UHPLC and a CaptiveSpray ion source connected towith nano-UHPLC MS/MS on the modified UHR-TOF. For peptide identification and quantitative analysis the MaxQuant software package was used (Nature Biotechnology 26, 1367 - 1372 (2008)).

For higher sensitivity at fast acquisition speed, ion extraction from the collision cell into the orthogonal acceleration of the TOF-analyzer was improved by using a novel collision cell design was used. Increased resolution without changing the effective flight path could be achieved with a modified reflectron. In addition, a faster detector (reduced width of individual ion signals) led to further improvements in resolving power.
Using an optimized detector digitizer combination, a threefold higher dynamic range was observed. However in complex samples, the dynamic range is also limited by the capability of the instrument to resolve nearly isobaric compounds. The performance improvements were analyzed in a label-free quantification experiment, evaluating in particular the number of quantifiable peptides over the entire dynamic range. As a defined model system for complex proteomics samples, a mixture of 48 standard proteins spanning a concentration range of five orders of magnitude (Universal Proteomics Standard, UPS-2, Sigma) was spiked into samples of 500ng E. coli, S.Cerevisiae (higher complexity) or human plasma (wider dynamic range) digests at a concentration of 1:2. During separation in with two hour gradients, the UPS-2 peptides could be quantified based on the MS full scans at levels from 500fmol down to the low attomole range. Similar analyses with background samples of higher complexity (S. cerevisiae) and wider dynamic range (human plasma) were carried out to further evaluate the benefit of the novel hardware features.

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Discovering new metabolic pathways in protozoan parasites using stable isotope labelled metabolomics

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Metabolomics is the global measurement of metabolites in a biological system. Established metabolomics methods allow relative quantitation of a broad range of metabolites, providing a snapshot of the metabolic state of the system under investigation. However, these methods do not reveal the connectivity of metabolites or the flux through metabolic pathways. A combination of high resolution untargeted metabolomics with stable-isotope labelling allows a system-wide analysis of metabolic flux and the subsequent discovery of active metabolic pathways within a cell.

The protozoan parasites *P. falciparum* and *T. brucei* are the causative agents of malaria and human African trypanosomiasis, respectively. These parasites salvage many nutrients from the host, or from the medium in cell culture experiments, but also possess numerous active metabolic pathways.

U-^{13}C-glucose labelling in cell cultures, combined with high-resolution mass specrotmetry-based metabolomics, allowed direct observation of the fate of glucose utilization in these protozoan pathogens. According to published models, *T. brucei* was thought to have a streamlined glucose metabolism, limited to glycolysis. However, our approach demonstrated incorporation of glucose-derived carbon into over 100 metabolites, revealing the presence of novel active metabolic pathways in these organisms.

Extensive post-glycolytic metabolism was also observed in *P. falciparum*, and stable isotope-labelled metabolomics analysis of knockout lines revealed a novel activity for the enzyme putatively annotated as branched chain keto-acid dehydrogenase (BCKDH) linking glycolysis to the TCA cycle.

Stable isotope labelling coupled with untargeted metabolomics enabled system-wide evaluation of active metabolic pathways in protozoan pathogens. Several novel pathways and enzyme activities were identified in *T. brucei* and *P. falciparum*, allowing significant extensions to previous models of glucose metabolism. This experimentally-derived, systems-based depiction of the active metabolic networks in these pathogens will assist in the elucidation of potential drug targets for the treatment of parasitic diseases.

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DISCOVERY OF VIRULENCE FACTORS IN *GIARDIA DUODENALIS* HOST-CELL INTERACTIONS

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Giardia duodenalis is a protozoan parasite and major contributor worldwide of diarrhoea and gastroenteritis in vertebrates, including humans. Giardiasis is characterised by proliferation of trophozoites in the small intestines, where parasites attach to the gut mucosa via a ventral disk resulting in infection pathology. Our experiment quantifies protein changes in trophozoites exposed to host soluble factors (HSF) from HT-29 intestinal epithelial cells (IEC), compared to the co-incubation with the cell-monolayer itself. This host-cell co-incubation permits attachment of trophozoites to host cells. All treatments, including the control, were incubated in serum-free DMEM for 6 hours at 37°C in 5% CO_2. Two complementary quantitative approaches; Tandem Mass Tags (TMT) labelling and label-free based on spectral counting approach were performed to quantify the differentially expressed proteins of biological triplicates between conditions. We identified a total of 1650 proteins, of which a small subset of 21 up-regulated and 14 down-regulated proteins were reproducibly differentially expressed, including up-regulation of known and putative virulence factors. Cathepsin B precursor, a confirmed immunomodulatory protein, was up-regulated in trophozoites exposed to HSF, but was not changed during co-incubation with host cells. Similarly, VSP antigenic switching was increased in trophozoites incubated with HSF compared to co-incubation. Several signalling proteins were up-regulated, in particular the Tenascins, during incubation with HSF. These results indicate that induction of virulence factors from several important protein families occurs independent of trophozoite attachment to host cells. Significantly, we observed equivalent up-regulation, in both HSF-exposed and co-incubated trophozoites, of the *Giardia duodenalis* cystatin homologue and protease inhibitor, which may represent a novel pathogenicity factor in *Giardia*.

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A platform for the structural characterization of glycans enzymatically released from glycosphingolipids extracted from tissue and cells

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Glycosphingolipids (GSLs) constitute a highly diverse class of glyco-conjugates which are involved in many aspects of cell membrane function and disease. The isolation, detection and structural characterization of the carbohydrate (glycan) component of GSLs are particularly challenging given their structural heterogeneity and thus rely on the development of sensitive, analytical technologies. In this study, neutral and acidic GSL standards were immobilized onto polyvinylidene difluoride (PVDF) membranes and glycans were enzymatically released using Endoglycoceramidase II (EGCase II), separated by porous graphitized carbon (PGC) liquid chromatography and structurally characterized by negative ion mode electrospray ionization mass spectrometry

(PGC-LC-ESI-MS/MS). This approach was then employed for GSLs isolated from 100 mg of serous and endometrioid cancer tissue and from cell line (107 cells) samples. Glycans released from GSL standards comprising of ganglio-, asialo-ganglio- and the relatively resistant globo-series glycans, using as little as 1 mU of enzyme and 2 μg of GSL. The platform of analysis was then applied to GSLs isolated from tissue and cell line samples and the released isomeric and isobaric glycan structures were chromatographically resolved on PGC and characterized by comparison with the MS2 fragment ion spectra of the glycan standards and by application of known structural MS2 fragment ions. This approach identified several (neo-)lacto-, globo- and ganglio-series glycans and facilitated the discrimination of isomeric structures containing Lewis A, H Type 1 and Type 2 blood group antigens and sialyl-tetraosylceramides. We describe a relatively simple, detergent-free, enzymatic release of glycans from PVDF-immobilized GSLs, followed by the detailed structural analysis afforded by PGC-LC-ESI-MS/MS, to offer a versatile method for the analysis of tumour and cell-derived GSL-glycans. The method uses the potential of MS2 fragmentation in negative ion ESI mode to characterize, in detail, the biologically relevant glycan structures derived from GSLs.

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YBX1 mediates partial EMT to promote tumourigenicity regulated by the extracellular environment

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Epithelial-mesenchymal transition (EMT) describes a morphogenetic program which confers mesenchymal cell properties such as reduced cell-cell contact and increased cell migration and invasion, to epithelial cells. Here we investigate the role of the pleiotropic transcription/splicing factor and RNA-binding protein nuclease-sensitive element-binding protein 1 (YBX1/YB-1) in inducing EMT and increasing the oncogenic potential of epithelial MDCK cells. Stable expression of YBX1 in MDCK cells (MDCK[YBX1]) induced partial EMT, including cytosolic relocalization of E-cadherin, increased cell scattering, and anchorage-independent growth. A critical feature of this study was the observation that MDCK[YBX1] cells were pro-oncogenic and established tumour xenograft growth, in contrast to parental MDCK/ MDCK[vec] cells. We further sought to identify the molecules that may confer these properties, and used an in-depth proteomic approach (UPLC Orbitrap Elite, MaxQuant workflow) to profile and quantitate (label-free quantitation) the cell lines from *in vitro* cell culture (cellular and secreted molecules), as well as the *in vivo* MDCK[YBX1] cell tumour xenograft directly. We reveal that YBX1 regulates the extracellular environment through expression levels of secreted/ extracellular proteins. The increased tumourigenicity of MDCK[YBX1] cells correlated with the identification of elevated levels of secreted factors (TGF-β, CSF-1, NGF, and VGF) and proteases (ADAM9 and ADAM17), known to enhance angiogenesis and cancer progression. Treatment of recipient 2F-2B endothelial cells with MDCK[YBX1] cell conditioned medium containing both soluble proteins and extracellular vesicles increased their motility. These findings contribute to our understanding of how YBX1 as an oncogenic enhancer mediates partial EMT in epithelial cells and promotes tumour angiogenesis via the elevated release of a new suite of secreted molecules.

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Discovery of protein lysine methyltransferases that act on translation elongation factor EF1α in *Saccharomyces cerevisiae*

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Lysine methylation is a post-translational modification with emerging proteome-wide functions. Like its counterpart, arginine methylation, it is known to affect protein-protein interactions, and this has implications for its role in modulating the dynamics of protein-protein interaction networks. To uncover the mechanisms by which lysine methylation modulates protein-protein interactions, it is essential to discover and characterise the enzymes which catalyse methylation. Through mass spectrometry-based screening of knockouts of four putative yeast protein methyltransferases, we have discovered three novel lysine methyltransferases that methylate the translation elongation factor EF1α – YGR001C, YNL024C and YLR285W. YGR001C trimethylates lysine 79, YNL024C monomethylates lysine 390 and YLR285W is responsible for the di- and tri-methylation of two novel sites – lysines 3 and 5. All three methyltransferases belong to the seven-beta-strand family of methyltransferases, with YGR001C being previously putatively assigned as a N(6)-adenine-specific DNA methyltransferase. N6AMT2, the human homolog of YGR001C, is likely to also be a lysine methyltransferase, as lysine 79 is known to be trimethylated in human EF1α. The structural contexts of the modifications suggest that they may have distinct roles in modulating the function of EF1α. Besides its canonical role in facilitating delivery of tRNA to the ribosome, EF1α is known to have a number of so-called 'moonlighting' functions – including regulating the actin cytoskeleton and export of tRNA – which are facilitated by its numerous protein interaction partners. Understood in this context, it is likely that these methyltransferases serve to control which of the many protein-protein interactions EF1α partakes in, and thereby have proteome-wide functional implications.

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Determination of Rab GTPases

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Isoprenylation is a form of posttranslational modification (PTM), which constitutes the attachment of isoprenoid groups to cysteine of the C-terminus of proteins. Two forms of isoprenylation are distinguished: Three isoprenoid units form the farnesyl group ($C_{15}H_{25}$) with a mass of 205 Da, whereas the geranylgeranyl group ($C_{20}H_{33}$) represents four isoprenoid units with a mass of 273 Da.

Prominent examples of isoprenylated proteins are the Ras and Ras-related proteins. Like other small GTPases, these proteins change between two notable conformational stages: the active membrane-associated GTP- and the inactive cytosolic GDP-form. In this context, the isoprenylation serves as a membrane anchor and allows the interaction between isoprenylated Ras and Ras-related proteins with their effector proteins.

All three isoforms of the Ras protein are farnesylated, and the members of the Rho protein superfamily are geranylgeranylated. The Ras-related superfamily of Rab proteins takes an exceptional position: Rab proteins receive two geranylgeranyl moieties from the Rab-geranylgeranyltransferase (Rab-GGTase). After double geranylgeranylation, Rab is integrated into the cell membrane and serves as a molecular switch in intracellular trafficking. Several Rab proteins are aberrantly expressed in tumors[1] but no Rab-specific drugs have been developed yet. Because of the essential isoprenylation for correct localization and function of Rabs, inhibitors of Rab-GGTase have been found[2]. Nonetheless, isoprenylated proteins are low-abundant in complex cell samples. This is why it is extremely difficult to detect them with mass spectrometry, despite sensitive measuring devices. Therefore, enrichment strategies are indispensable.

We have established an elutropic series by using liquid chromatography-mass spectrometry (LC-MS), which discriminates farnesylated, singly and doubly geranylgeranylated peptides. Furthermore, we investigated characteristic gas phase reactions of isoprenylated peptides during ionization and fragmentation.

These results pave the way for future studies on e.g. prenyltransferase inhibitors without further enrichment steps.

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A systematic label-free quantitative approach to discover novel substrates of E3 ubiquitin ligases.

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Untargeted label-free quantitative proteomics has become an increasingly powerful methodology that can provide vital quantitative information, either relative or absolute, on large parts of the expressed proteome. Contrarily to label-based methodologies, label-free quantitation is applicable to any kind of sample and, in principle, has no limitation on the number of samples that can be compared. These advantages make label-free quantitative proteomics the method of choice for analysis of clinical samples, primary cells and tissues.

In this study we set out to identify novel substrates for the family of Membrane-associated RING-CH (MARCH) E3 ubiquitin ligases *in vivo*. Regulation of cell surface receptors is essential for the maintenance of the cell proteostasis, and ubiquitination at the plasma membrane has been observed to be a fundamental post-translational mechanism regulating a wide variety of surface proteins, including immune receptors. The MARCH ligases are a subfamily of the RING E3 ligases originally identified in viruses to be involved in immune evasion strategies. Numerous in vertebrate studies suggest that the MARCH ligases' main role is to regulate trafficking, surface expression and turnover of immune receptors, however most of their substrates remain unknown.

In order to identify novel surface targets of the MARCH ligases we developed a plasma-membrane enrichment strategy from primary antigen presenting cells (DCs), purified from wild-type and MARCH-deficient mouse spleens. Quantitative analysis of LC-MS/MS data was performed using MaxQuant for peptides/proteins identification and for feature detection, while methods for the quantitative analysis and statistics were developed in house. Through this approach we were able identify potential novel targets for most of the MARCH ligases as well as to confirm previously characterised ones. With this work we have established a robust methodology to systematically identify potential targets of ubiquitin ligases *in vivo*.

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Comprehensive protein sequence databases and the advent of personalized sequence databases

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"Which sequence database should I use for my proteomics analysis?" is a question that is perhaps not asked often enough. Reference protein sequence databases (such as Ensembl, UniProtKB, NCBInr or LudwigNR) all have their own advantages, disadvantages and quirks. Based on 3 different proteomic applications from protein characterisation, pathogen detection and OMICS integration (RNASeq and Proteomics), I will illustrate the advantages and disadvantages of these publically available reference sequence databases and hopefully shed some light and provide insight into the questions that should be asked, which ultimately of course depends on the experiment at hand and the biological questions being asked. More recently, the integration of datasets sourced from different technologies (such as RNASeq and Proteomics) has become a reality. Based on 2 different

projects I will share some insight into the benefits and methodology involved in facilitating this integration and of course the pitfalls associated with this task.

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Sialic acid involvement in bacterial binding to different body fluids

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One of the key initial processes involved in bacterial infection is the attachment to host cell receptors, typically glycans (sugars) conjugated to proteins or lipids on epithelial cell surfaces. Accordingly, many pathogens have developed adhesins or lectins that attach to specific glycan epitopes characteristic of the host cells they target. In turn, we propose that the host provides an ingenious innate defence mechanism that uses the glycoproteins in their secreted fluids to competitively bind to the bacterial pathogens to prevent infection.

Our research has demonstrated that bacteria bind differentially to glycan moieties on secreted glycoproteins from human tears, milk, saliva and sweat, potentially providing decoys for selective adhesion and clearance of pathogens as the secretory fluids wash the epithelial cell surface. Here we demonstrate the differential involvement of sialic acid in the adhesion of pathogenic and commensal bacteria to secreted glycoproteins in these human secretions. Our techniques include a 96 well plate bacterial adhesion assay in which the glycoproteins bind to the PVDF membrane in the wells through hydrophobic interactions, leaving the glycan molecules exposed on the membrane surface. The presented glycans are then probed with fluorescently-labelled bacteria and bacterial binding quantified using a fluorescent plate reader. The importance of terminal sialic acid in the bacterial–secreted glycoprotein interaction was determined by detecting any change in the bacterial binding following neuraminidase treatment.

The work demonstrates a natural mechanism by which sialic acid contributes to the attachment of pathogenic bacteria to the glycoproteins secreted in a diverse range of body fluids and suggests there is a glycan-mediated protective process against infection in all human secretions.

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Plasma biomarkers for the detection of human growth hormone abuse in sports

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Human growth hormone (hGH) is a naturally occurring, 191 amino acid peptide hormone secreted by the pituitary gland with anabolic and growth-promoting activity. Since the increased availability of rhGH in the late 1980s for the treatment of hGH-deficient patients, rhGH has become a drug of abuse in sport and it is prohibited by the World Anti-Doping Agency (WADA). Currently, there is no method available that can clearly discriminate between GH user and nonuser due to difficulties in direct differentiation between native and recombinant iso-forms of hGH, short half-life in circulation, and not enough biomarkers reported. We have identified new GH-depended plasma biomarkers by two-dimensional difference gel electrophoresis (2-D DIGE) and Isobaric tags for relative and absolute quantitation (iTRAQ) analyses which have longer half-life than rhGH itself. We have validated the identities of candidate markers by using orthogonal methods such as western blotting and selected reaction mass spectrometric (SRM) analysis. By combining bio-markers reported by others and new markers that we have identified in our work may provide us an opportunity to develop a better rhGH abuse detection method in sports or monitoring changes in plasma proteome after GH-administration in clinical trials.

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Redox modifications of cysteine in the liver of type 2 diabetes mellitus

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Reactive oxygen species (ROS) and associated redox modifications have normal physiological roles in signalling but are also implicated in a number of pathologies. Oxidative stress associated with type 2 diabetes mellitus (T2DM) has been attributed to increased generation/reduced clearance of ROS in insulin sensitive tissues including the liver[1]. During oxidative stress, proteins undergo redox modifications through the thiols of cysteine, which may alter structure, functionality and signalling pathways. To determine proteins of the liver which undergo redox modifications with T2DM, we investigated a number of associated metabolic disorders using redox proteomics. Rats were subjected to a high fat diet (HFD) to induce peripheral insulin resistance, as well as streptozotocin (STZ) to create β cell dysfunction, thereby generating the T2DM pathology. Rats treated with only HFD or STZ were utilised to analyse the pre-diabetic state. To enrich low abundance redox modified peptides, thiol disulfide exchange was utilised for reversibly modified cysteines[2] with quantitation by iTRAQ. Strong cation exchange was used to select for irreversibly modified cysteines[3], combined with label-free quantitation. Enriched and non-captured fractions were subjected to 2D-LC to increase depth of coverage before identification by tandem mass spectrometry. Using this approach, the TripleTOF 5600 (AB Sciex) identified 8038 redox-modified sites in the liver proteome, with over 2223 sites quantified in response to T2DM (HFD+STZ). Oxidative modifications to proteins which maintain redox balance suggests dysregulation, contributing to oxidative stress in T2DM. Observed changes in enzymes involved in glucose metabolism suggest a role of redox modifications in glucose

utilisation/production. Modifications to enzymes in fatty acid metabolism correlate with the hyperlipidemia associated with T2DM. Many proteins showed increased levels of irreversible oxidative modifications contributing to protein damage. Changes in the redox status of these proteins could be indicative of these protein species prone to oxidative damage, contributing to pathogenesis or a level of cellular regulation in response to the metabolic disturbance.

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Toward a better understanding of glycosylation machinery by subcellular proteomics and glycomics

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Publish consent withheld

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Paucimannosylation in human neutrophils: Insights into the biosynthesis and immunological role of a novel type of protein *N*-glycosylation

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The biosynthetic pathway of mammalian protein asparagine (*N*)-glycosylation is known to produce three classes of *N*-glycans of the high mannose, hybrid and complex type that are abundantly expressed on glycoproteins. A fourth class of *N*-glycosylation known as paucimannosylation, defined as the addition of a full or partial chitobiose core to asparagine residues, is considered to be a glycosylation feature of invertebrates. However, recent literature has reported the presence of paucimannosidic proteins in humans and in mice during inflammation-related conditions, suggesting a potential immunological link to these novel glycoproteins in mammals. Given the importance of neutrophils in innate immunity, we here perform an *in vitro* based investigation of the biosynthesis and potential immunological functions of protein paucimannosylation in human neutrophil-like HL60 cells. Moderate to strong co-localisation of paucimannosidic epitopes, β-hexosaminidase A (Hex A) and azurophilic granule resident myeloperoxidase using immunofluorescence indicates involvement of Hex A in the synthesis of protein paucimannosylation, suggesting an azurophilic-specific localisation of paucimannosidic proteins in neutrophils. Additionally, HL60 and *P.aeruginosa* co-culture and the use of mass spectrometry showed that the azurophilic compartments of the neutrophil can be mobilised upon virulent stimuli, resulting in a secretion of paucimannosidic proteins into the extracellular milieu. Finally, we show that human proteins carrying these novel glycoepitopes display significant bacteriostatic activities towards specific *P. aeruginosa* strains under biologically relevant concentrations, as shown by the effect of purified paucimannosidic proteins on bacteria growth. Taken together, we here provide evidence supporting functionally important, previously un-described, roles of protein paucimannosylation in human neutrophils.

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Bioinformatics approaches to the proteomics of secreted proteins in *Arabidopsis thaliana*

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Publish consent withheld

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Metalloproteomic profile of natively purified alpha synuclein from blood and brain, including the identification of the associated protein truncations by mass spectrometry.

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Alpha synuclein is an abundant neurological protein that has been implicated in a number of neurodegenerative diseases. It has been proposed that the mechanism by which this protein elicits its effects in these diseases is through metal-protein interaction causing conformational changes which results in aggregation and oxidative stress. However the exact pathological role of alpha synuclein is yet to be elucidated and the interactions between it and metals have only been shown using recombinant proteins. Most recombinant protein techniques lack the machinery for incorporating post-translational modifications common to eukaryotic proteins, including chaperones for delivering copper. To investigate the metal status of native alpha synuclein we have purified it from both human erythrocytes and human brain tissue using non-denaturing techniques. Using size exclusion chromatography coupled to inductively coupled plasma – mass spectrometry we were able to directly determine the metal status of alpha-synuclein. Results indicated that alpha synuclein does not bind significant amounts of copper, iron or zinc, even when these metals were added to the protein in excess. In addition to this we used intact mass spectrometry to identify alpha synuclein, which was present in different truncated forms in the blood and the brain. Based on these results native alpha synuclein purified using these techniques is not a metalloprotein and can is present in multiple truncated versions in the blood and brain.

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Detection of oxidative modified proteins in rice plant

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Plant is sessile as it is constantly being exposed to environment stresses. These stresses can cause the build-up of reactive oxygen species (ROS) in plant cell that will cause oxidative damage on the DNA, lipid and also proteins. There are two types of oxidative modified protein, one being reversible and another one could lead to permanent damage to the plant cell. The reversible proteins also usually act as signalling proteins. Hence, detection and identification of these proteins is prime target for crop improvement. In this study, 5-Iodoacetamidofluorescein (5'IAF) that competes with hydrogen peroxide was used to identify oxidative modified cysteine proteins following the application of oxidative stress. This approach enables the identification of six groups of proteins that regulate the plant stress mechanisms. The six groups of proteins include L-ascorbate peroxidase redox protein, S-adenosylmethionine synthase and cysteine synthase that regulate amino acid metabolism, salt stress root protein RS1, photosynthesis proteins and seed storage proteins. The interaction of the key proteins observed in this study with the downstream of metabolites in combination with other phenotypes will provide a full picture of the role of these proteins in stress adaptation. The findings provide useful information as to production of stress tolerance plant in the future.

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Predicting Protein Abundance based on Mass Spectrometry using Machine Learning

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Motivation:

Protein quantification has long been an interesting and paramount area in proteomics field. MaxQuant, currently one of the most commonly used softwares for protein quantification, employs state-of-the-art peptide intensities based methods. Using state-of-the-art protein quantification methods such as iBAQ, it has been shown that the accuracy of protein quanification is dependent on its abundance such that highly abundant proteins can be much more accurately quantified compared with low abundance proteins.. Other peptide ion intensities based quantification methods have the same problem. (Wilhelm, et al. 2014)

Protein peptide mass spectrometry intensities have been verified to be correlated with its protein abundance and chemical and biophysical features (Scherbart, et al. 2009). In this study, we development a machine learning to model the relationship between chemical and biophysical peptide features and its protein abundance in an attempt to improve MS-based protein quantification accuracy

Results:

Machine learning models including Support Vector Machine and Artificial Neural Networks (including Deep Learning Models) with various topologies was developed and used to model a set of 690 chemical and biophysical features including AAIndex features (Kawashima, et al 2008) associated with each peptide.

First, our machine learning models are trained on AQUA data of U2OS (4490 proteins), and the pearson correlation coefficience R between our predicted protein abundance using cross validation and AQUA abundance is substantially higher than that between AQUA and iBAQ abundance ($R_{pearson}$ increases from 0.806 to 0.90578). This indicates our developed protein and peptide feature set can assist improving the accuracy of calculating protein abundance via machine learning models.

Second, we compared our peptide feature-based protein abundance prediction model with MaxLFQ (Cox, et al. 2014) using, a dataset consisting of proteins spiked in with known ratios of SILAC labelled protein. Our model was trained using a set of proteins where the SILAC ratio accurately corresponded with the expected ratio. This model was then applied in predicting abundance of all the proteins (4446 proteins) in the dataset. To date, the predicted protein abundances also shows the potential of outputing protein abundance ratio distribution better than MaxLFQ and our research is continuing for further optimizing the performance of the models.

Conclusion:

Our study shows that by considering peptide features when computing protein abundance estimation from MS-based data the accuracy of the measurement can be improved. This has important implications particularly in label free protein quantification and studies to correlate gene expression and protein abundance.

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A proteomic investigation for detection of early stage CRC biosignatures

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The current methods widely deployed for colorectal cancer (CRC) screening are grossly inadequate on sensitivity and specificity ground. Cancer specific biomarkers are found in low concentrations (pg- ng/mL) in plasma as a result of structural changes in the microenvironment of cancer tissues followed by dilution in plasma. This study was designed to meet the longstanding unmet clinical need for cancer screening and surveillance using minimally invasive techniques. Expression of 92 potential biomarkers were measured from clinically stage CRC (Duke's A, B, C, D) and control (i.e., unaffected) EDTA plasma samples utilizing PEA based Proseek® Multiplex Oncology I kit. A duplicate set of samples were analysed by Bio-Plex Pro™ human cytokine 27-plex immunoassay kit. Expression of CEA (a diagnostic biomarker for CRC) was found to be significantly high in malignant stage while IL 8 and prolactin had significant level of expression between control, benign and malignant stages. Additional experiments are being conducted on the same set of plasma using commercially available MARS 14 followed by in house immune based depletion system. The aim is to detect low abundance novel biomarkers using state-of-the-art LC-MS instrumentation housed in Australian Proteome Analysis Facility. In addition, uHPLC based analysis is being conducted on these samples to identify any fluctuation trend in kynurenine pathway in various cancer stage. The outcomes from this study will be followed up with a subsequent investigation where expression of 17 new proteins will be measured by PEA technology as well as statistical correlation will be established between the expression and clinical data. We believe these findings will enable to establish new, improved and volume-sparing plasma biomarkers/biomarker signature panels. The detailed results will be presented at the upcoming Lorne Proteomics conference which would specifically provide useful information to researchers about a longstanding unmet clinical need for early stage CRC detection.

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A workflow to identify targets of the E3 ubiquitin ligase Nedd4 involved in controlling neural crest cell fate.

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Advances in diglycine remnant affinity enrichment techniques have seen ubiquitylation studies enjoy a significant amount of attention in recent proteomic research. Here we report our findings from experiments aimed at identifying targets of the HECT E3 ubiquitin ligase, Nedd4. We have previously shown Nedd4 plays a critical role in neural crest cell development. Our studies in Nedd4^{-/-} mice reveal striking defects in the development of neural crest cell derived tissues, including craniofacial and cardiac. In combination with *in vitro* data, these studies implicate Nedd4 as an important factor in the maintenance of neural crest cell identity, survival and/or stemness. To begin addressing how neural crest cells are affected by loss of Nedd4, we needed a workflow to identify targets. To this end, we have carried out siRNA knockdown of Nedd4 in a SILAC labeled neural crest cell line and used a diglycine remnant enrichment strategy to reveal diglycine peptides that are less abundant following Nedd4 knockdown compared to controls. We compare these data to total protein abundance data gathered from samples by LC-MS/MS prior to diglycine remnant enrichment, to discern changes specifically in diglycine peptide abundance from changes in total protein. And finally, we cross check our proteomic data to expression data obtained from the same experimental model to better distinguish whether changes in protein abundance arise from changes in stability or from changes in gene expression. Taken together, we present a workflow for the identification of protein targets of E3 ubiquitin ligases and reveal a number of interesting and novel putative Nedd4 targets.

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Spectral Libraries for SWATH: Establishing a Comprehensive Database for Cancer Research

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SWATH represents an important development for establishing a comprehensive LC/MS based quantitative strategy for cancer based proteome projects. The approach currently relies on the development of spectral libraries containing both product ion data and retention time information, and provides the basis for extensive data-independent quantitation. Although significant progress has been made recently towards mapping the human proteome, spectral libraries remain an important target resource for proteomics.

Here we report on the assessment of new 6600 Triple TOF technology in providing extended proteome coverage and the establishment of a comprehensive spectral library for cancer research. We have observed a two-fold increase in protein coverage using a 6600 Triple TOF compared with a 5600 Triple TOF, and now detect over 20,000 distinct peptides from 3000 proteins in a single two-hour 1-dimension chipLC/MS analysis. Utilising this additional capacity in proteome coverage, we have developed a comprehensive spectral library for SWATH using a variety of human cancer cell lines, including melanoma, colorectal, thyroid, cervical and transformed embryonic kidney cells. We demonstrate the utility of this extended cancer database by characterising quantitative differences of each cancer cell line via SWATH.

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Understanding the molecular mechanisms of water stress tolerance induced by *Piriformospora indica* in barley

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Piriformospora indica is a mutualistic root endophytic fungus, which transfers several benefits to hosts including enhance plant growth and increase yield under both normal and stress conditions. It has been shown that *P. indica* root-colonization enhances water stress tolerance based on general and non-specific plant-species mechanism. To better understand the molecular mechanism of *P. indica*-mediated drought stress tolerance, we designed a set of comparative experiments to study the impact of *P. indica* on barely plants cultivar "Golden Promise" grown under different drought levels [Filed capacity (F.C.), 25% F.C and 50%]. *P. indica* enhanced root and shoot biomass of colonized plants under both well-watered and water-deficit conditions. Proteome analysis of *P. indica*-colonized barley leaves under well-treated and water-deficit conditions resulted in detection of 726 reproducibly protein spots. Mass spectrometry analysis resulted in the identification of 45 differentially accumulated proteins involved in photosynthesis, reactive oxygen scavenging, metabolisms, signal transduction, and plant defense responses. Interestingly, *P. indica* increased the level of proteins involved in photosynthesis, antioxidative defense system and energy transport. We propose that *P. indica*-mediated drought stress tolerance in barely is through photosynthesis stimulation, energy releasing and enhanced antioxidative capacity in colonized plants.

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Mass Spectrometry Analysis of Endometrial Cancer

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"Endometrial cancer with regional lymph node metastasis has a high mortality rate. Lymph node metastasis is therefore a crucial factor in the prognosis and choice of treatment for patients with endometrial cancer. Based on the assumption that metastasis is mainly determined by properties of the primary tumor and its interaction with the surrounding structures we aim to explore the possibility of predicting the presence of metastasis based on the molecular features of the primary tumor.

A tissue cohort comprising of endometrial primary tumors with metastasis (n=41) and without lymph node metastasis (n=51) was analysed to identify proteins associated with regional lymph node metastasis. For this, two complementary methods MALDI Imaging and Label free quantitative proteomics were used. Though MALDI-IMS allows the analysis of hundreds of peptides and lipids simultaneously, MALDI is not considered quantitative. Therefore, quantitative proteomic profiling by label free analysis will be used in conjunction with MALDI-IMS to identify proteins and peptides that are differentially expressed in primary tumours and have been metastasized. Our preliminary data of tryptic peptide imaging on FFPE tissue micro arrays indicates the metastatic potential of the primary tumor. Using the label free quantitative approach, we have identified three proteins which are differentially expressed between primary tumors with and without lymph node metastasis. In conclusion, we expect that the results from this study will provide crucial new tools to assist in the diagnosis and prognosis of Endometrial cancer and would prevent overtreatment of patients, whose primary tumor does not have metastatic potential."

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Identification of diagnostic biomarkers to improve the management of diabetic related foot ulcers

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Diabetic related ulcers are a common and severe complication of diabetes that can lead to severe health complications and prove fatal if mistreated or undiagnosed. Currently, there is lack of understanding regarding the underlying biochemical and physiological processes involved during chronic wound healing. The objective of this project was to understand and investigate the underlying factors in the wound environment that could be used to distinguish healing from non-healing diabetic foot ulcers. Bottom up proteomics was performed to analyse healing and non-healing representative pooled samples using SDS-PAGE and LC-MS/MS by AB SCIEX TripleTOF® 5600 System. The data analysis identified more than 400 proteins each from healing and non-healing samples, and 15 and 16 proteins unique to healing and non-healing respectively. A semi-quantitative approach involving spectral counting revealed more than 200 differentially regulated proteins in the samples. Proteins were grouped based on different functional properties and oxidative stress related proteins were selected for further analysis as there is growing evidence to suggest their vital role in the pathogenesis of diabetes related ulcers. The top 6 abundant proteins, catalase, peroxiredoxin 1, peroxiredoxin 2, superoxide dismutase-2 (SOD-2), S100-A9 and myeloperoxidase (MPO), were chosen on the basis of semi quantification results for further validation using enzyme-linked immunosorbent assay (ELISA).

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Tracking the intricate dynamics of antigen and epitope kinetics during virus infection

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The CD8+ T cell response to virus infection is dependent upon recognition of peptide epitopes complexed to MHC molecules and presented on the surface of infected cells. Whilst the mechanisms underlying this processing pathway are known, there is a paucity of quantitative information regarding the dynamics of presentation and how this may impact upon the magnitude of the T cell response. Here we have used targeted mass spectrometry to investigate the display of epitopes during vaccinia virus infection of three different cell types; namely *in vitro* fibroblasts and dendritic cells, and *ex vivo* bone marrow-derived dendritic cells. LC-MRM was used to simultaneously track the kinetics of 47 vaccinia-derived epitopes presented to the immune system, incorporating stable isotope-labeled peptides in order to provide measures of absolute abundance, whilst data-independent SWATH-MS was used to track antigen levels and profile virus proteome expression. The CD8+ response to each epitope was measured in parallel from infected mice in order to determine the immunodominance hierarchy each peptide elicits.

We reveal that the nature of vaccinia epitope display is highly diverse during infection, with peptide levels spanning many orders of magnitude. Further, whilst the relative kinetic of each epitope was remarkably similar across each cell type, abundance levels were found to vary considerably, with notably diminished levels on fibroblasts. Antigen expression and epitope kinetics were tightly coupled, as is predicted in order to engender a rapid T cell response to infection. However, correlations between epitope abundance and CD8+ response magnitudes remain difficult to predict, despite here using *ex vivo* dendritic cells as a model of *in vivo* infection. This study represents an advancement in our understanding of virus infection in the context of the immune system and will be necessary to drive further investigation into factors that can tailor immunity to pathogens for therapeutic benefit.

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Depolarisation-induced reprogramming of the presynaptic phosphoproteome mediates changes in neurotransmitter release

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Learning and memory result from synaptic plasticity - the ability of synaptic transmission to be modulated over time in response to stimuli. In contrast to the well-studied postsynaptic changes, presynaptic change in neurotransmitter release (exocytosis) from synaptic vesicles (SVs) is a mechanistically less understood form of synaptic plasticity. Exocytosis is regulated by phosphorylation, and the phospho-status of several exocytic proteins affects transmitter release. Exocytosis is followed by synaptic vesicle endocytosis which is controlled by proteins that are rapidly dephosphorylated upon nerve stimulation. We have used large-scale quantitative phosphoproteomics to study proteins from isolated nerve endings from rat brain (synaptosomes) that were at rest, depolarised for 10 sec. using KCl, or depolarised and allowed to recover for up to 15 min after depolarisation. After stimulation, the synaptosomes were lysed, subjected to protein precipitation and digested using trypsin. Phosphopeptides were enriched and fractionated using a combination of TiO_2, sequential elution from IMAC (SIMAC) and hydrophilic interaction liquid chromatography (HILIC) (collectively abbreviated TiSH). Peptides were analysed by LC-MS/MS on an LTQ-Orbitrap Velos/Elite and a 5600 TripleTOF instrument. The results show a prolonged, global reprogramming of the synaptic phosphoproteome, with an overrepresentation of exo- and endocytic proteins showing altered phosphorylation levels, and we therefore hypothesise that the initial short-term depolarisation changes the potential for subsequent neurotransmitter release on a much longer time-scale. Neurotransmitter release experiments are currently being performed and preliminary data seem to confirm the hypothesis – showing that the level of glutamate release from synaptosomes at a second stimulation 15 min after the initial one depends on the strength of the initial stimulation. In conclusion, our results indicate that depolarisation-induced phosphorylation changes in the pre-synaptic proteome constitute a previously undescribed form of pre-synaptic plasticity.

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On the hunt for mitochondrial proteolysis: Profiling changes in the mitochondrial N-terminal proteome during bacterial infection

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Mitochondria mediate key cellular processes, including energy generation and cell death. Although mitochondrial proteases are important regulators of these processes, tools to study global proteolysis within mitochondria have not been developed.[1,2] Our understanding of mitochondrial dynamics could be greatly improved with a proteomics technique that also identifies proteolytic events.

We have developed a new approach called 'mito-TAILS' that quantitatively profiles the mitochondrial proteome and proteolytic events. Mito-TAILS combines mitochondrial enrichment, stable isotope labeling by amino acids in cell culture (SILAC),[3] and an amino (N)-terminal proteomics technique called terminal amine isotopic labeling of substrates (TAILS).[4]

In this study, mito-TAILS was used to characterize changes in the mitochondrial N-terminal proteome in response to infection with enteropathogenic *Escherichia coli* (EPEC). EPEC possesses several virulence factors that target mitochondria; they are imported, cleaved, and contribute to apoptosis of infected human cells via the mitochondrial pathway. Human epithelial (HeLa) cells were grown in either light or heavy isotope-coded SILAC medium and either infected with wild-type EPEC or mock infected in parallel. Cells were harvested, pooled, and homogenized. Mitochondria were then isolated from 95% of the cell homogenate; 5% was used for a complementary whole cell analysis. N-terminal proteomes were quantitatively compared using TAILS[5] and tandem mass spectrometry. Data from three biological replicates and two cellular fractions were analyzed using programs developed in our lab.

Proteins from mitochondrial cellular compartments in the Gene Ontology were significantly enriched in the mitochondrial fractions from mito-TAILS. We identified changes in the overall abundance of mitochondrial proteins, changes in mitochondrial protein import, and known apoptotic events. Furthermore, novel proteolytic events were identified in mitochondrial proteins that regulate apoptosis and protein import. By profiling the cellular proteome alongside the mitochondrial proteome, this technique permits a more comprehensive investigation of cell death events in both mitochondria and cytoplasm.

Mito-TAILS is a valuable approach to study mitochondrial proteome dynamics and proteolysis. To our knowledge, this is the first application of terminal proteomics to study mitochondria during infection and the first study of the mitochondrial proteome during infection. This method could easily be applied in other disciplines, including the study of mitochondrial diseases.

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Enhancing discovery and quantitative proteomics by use of online multiphase chip LC fractionation

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Online strong cation exchange (SCX) coupled with reversed phase (RP) mass spectrometry (MS) also known as MudPIT (multi-dimensional protein identification technology) is widely used in discovery-based proteomics. We combined MudPIT with selected reaction monitoring (SRM) MS to avoid off-line sample handling and showed a significant boost in signal response (90% increase).[1]

Encouraged by these findings, we developed chip-based SCX coupled RP columns to achieve higher column-to-column reproducibility and greater robustness of automated LC-MS/MS workflows. Technical proofs were established using cell lysates and plasma (non-immunodepleted) digests (5 µg). Five-step salt-based fractionation followed by 1 h acetonitrile gradients revealed significant peptide identification increases compared with 1 h RP-LC-MS/MS (5 µg, ~80% increase) and more accumulated unique peptides compared with 5x 1 h RP-LC-MS/MS runs (25 µg, ~10% increase). Multiphase separated samples demonstrated remarkable elution consistency (less than 6% in more than 1 fraction in cell lysates, and less than 13% in plasma).

Highly reproducible separation enabled multiphase chip integration into high resolution multiple reaction monitoring (MRM-HR) applications. Fraction-specific and retention time scheduled peptide lists (503 peptides from 173 plasma proteins) were generated. All targeted peptides were quantifiable in multiphase MRM-HR while 16% (85 peptides) were undetectable in

conventional MRM-HR and demonstrated 70% mean peak area increases. Interestingly, predominantly early eluting peptides demonstrated greatest increases (>10-fold).

Multiphase fractionation was also beneficial for spectral library generation in data independent quantitation (SWATH-MS). Five-step fractionation of thyroid cancer cell lysates led to spectral library generation for 3000 proteins. Analysing SWATH-MS acquisitions of same lysates resulted in reliable quantitation of 85% of all proteins in the library.

Using multiphase fractionation with SWATH-MS to analyse 10 melanoma cells with different MAPK pathway mutations demonstrated (principle component analysis) explicitly segregation of MEK inhibitor sensitive cells from MEK insensitive cells with distinct marker proteins for these phenotypes (confirmed by SRM-MS).

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Characterisation of glycosylation of the Newcastle disease virus haemagglutinin-neuraminidase surface glycoprotein

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Newcastle disease virus (NDV) is an avian virus belonging to the family *Paramyxoviridae* and the causative agent of Newcastle disease. The desire to study NDV is due not only to the significant economic impact it has on the poultry industry worldwide, but also its potential use as an oncolytic agent and vaccine vector for human and animal use.[1-3] Of great importance to NDV is the attachment surface glycoprotein, haemagglutinin-neuraminidase (HN). HN recognises and binds sialylated structures on host cells and facilitates virus-cell membrane fusion. It has been shown that the N-linked glycans present on HN can modulate the ability of the virus to infect cells and stimulate the host immune system.[4] However, characterisation of site-specific glycan occupancy and heterogeneity remains one of the few unexplored areas related to NDV HN. Accordingly, mapping the repertoire of glycans from HN may help elucidate mechanisms of infectivity and immune evasion. Not only with respect to NDV, but also within closely related viruses that cause disease in humans, such as parainfluenza viruses. Liquid chromatography-MS/MS strategies were implemented to structurally characterise the digested glycoprotein. Dissociation techniques included higher collision energy dissociation (HCD) and electron transfer dissociation (ETD) using high mass accuracy Orbitrap mass analysis and collision induced dissociation (CID) with linear ion trap mass analysis. Examination of NDV HN revealed considerable diversity amongst the N-linked structures, with up to sixty-three glycoforms present at one site. These included high mannose, fucosylated, sialylated and mono- and disulphated N-linked glycans. In addition, a novel sialylated O-linked glycan was also discovered, which may have implications in the functional interactions of the protein.

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Validation of mesenchymal stem cell transcripts assembled from RNA-Seq using proteomics data

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Alternative splicing of mRNA diversifies the function of human proteins, with tissue- and cell-specific protein isoforms being the most difficult to validate. While transcriptomic experiments enable the detection of many alternatively spliced transcripts, it is not known if these transcripts have protein-coding potential. We recently published the PG Nexus pipeline[1], which facilitates high confidence validation of exons and exon-exon junctions of spliced transcripts by integrating transcriptomics and proteomics data. In this study, we applied PG Nexus towards the analysis of an undifferentiated human mesenchymal stem cell line and compared the number of protein isoforms validated using different protein sequence database, including public online databases and RNA-seq derived databases. With significant overlaps with other databases, we identified 8,011 exons and 3,824 splice junctions from 2,379 genes with the Ensembl database. The Ensembl database consistently outperformed the other data sources, but predicted open reading frames from RNA-seq derived transcripts were comparable, with only 6 less splice junctions validated. Using proteotypic and isoform-specific peptides, we validated 462 protein isoforms and a higher number is possible if we included multiple proteotypic peptides. Multiplexing proteotypic peptides in SRM assays or similar experiments will increase the confidence and coverage of protein isoform validation experiments.

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Virulence factors of pathogenic *Escherichia coli* are injected into human cells and affect the human cellular N-terminal proteome

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Enteropathogenic *Escherichia coli* (EPEC) infects human intestinal cells and causes severe diarrheal disease worldwide. During infection, EPEC utilizes a syringe-like type III secretion system (T3SS) to inject virulence factors directly into the cytoplasm of the infected human cell. Identifying the functions and mechanisms of ≥23 T3SS effector proteins is a substantial challenge. Characterizing human cellular proteome dynamics during infection could substantially contribute to our understanding of how T3SS effectors contribute to EPEC-mediated disease.

This study aims to characterize T3SS-mediated alterations in the human proteome using an N-terminal proteomics approach. Proteases regulate human cellular processes relevant to infection, including inflammation, innate immunity, and cell death. N-terminal proteomics enriches proteolytic events, facilitating the study of protease activity and dysregulation. This study may reveal how these and other global pathways are affected by T3SS effectors and may identify novel substrates of T3SS proteases.

Cultured human epithelial cells were infected for 1.5 hours with wild-type EPEC or a mutant unable to secrete T3SS effectors. The N-terminal proteomes of infected human cells were quantitatively compared using terminal amine isotopic labeling of substrates (TAILS) and tandem mass spectrometry on an Orbitrap Velos mass spectrometer.[1] Data from three independent experiments were analyzed by Mascot and quantified by MaxQuant.

We identified 2112 unique modification-specific N-terminal peptides from 1359 unique proteins at 1% FDR. Preliminary data indicate T3SS-dependent proteolysis of proteins involved in known T3SS-mediated pathways, including innate immunity and cytoskeletal regulation, as well as novel proteolytic events in proteins known to interact with EPEC T3SS effectors. Finally, novel proteolytic events in beta-actin may provide further mechanistic details on how the T3SS mediates host cytoskeletal rearrangements during infection, a hallmark of EPEC infection.

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Sirtuin 4 is a lipoamidase regulating the activity of the pyruvate dehydrogenase complex

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Sirtuins (SIRTs) are a critical family of seven mammalian nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes which govern genome regulation, metabolism, and aging. SIRTs display widespread subcellular distributions; SIRT1, SIRT6, and SIRT7 are nuclear, SIRT2 is predominantly cytoplasmic, and SIRTs3-5 are mitochondrial. Despite all SIRTs containing a conserved deacetylase domain, only SIRTs1-3 show robust deacetylation activity, while mitochondrial SIRT4 and SIRT5 have little to no deacetylase activity. Emerging evidence has revealed that several SIRTs can hydrolyze alternative lysine modifications more efficiently than acetyl. Specifically, SIRT5 preferentially desuccinylates and demalonylates protein substrates, while SIRT6 can hydrolyze long-chain fatty acyl lysine modifications. A catalytic activity for SIRT4 has not been identified, however, here, we identify that SIRT4 is a robust lipoamidase. SIRT4s catalytic efficiency for lipoyl- and biotinyl-lysine modifications is superior to its deacetylation activity. The pyruvate dehydrogenase complex (PDH), which converts pyruvate to acetyl-CoA, is a biological substrate of SIRT4. We demonstrate that SIRT4 can enzymatically hydrolyze the lipoamide cofactors from the E2 component dihydrolipoyllysine acetyltransferase, and diminish overall PDH activity. For over 40 years, PDH has only been known to be regulated by phosphorylation of its E1 component. However, we now reveal its regulation by SIRT4, and establish SIRT4 as the first mammalian cellular lipoamidase, and a crucial guardian of metabolism.

GLYCOPEPTIDE ANALYSIS OF PENTAMERIC AND HEXAMERIC IMMUNOGLOBULIN M

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Human immunoglobulin (Ig) M is the largest antibody that exists naturally as a pentamer (with J-chain) or a hexamer form in blood circulation. They are the first antibodies to respond when a new or self-antigen is detected. A subset of circulating IgM, termed natural IgM, is responsible for the quality control of healthy cells and has reported anti-cancer therapeutic capabilities.

To this end, a monoclonal natural IgM that targets cancer cells has been recombinantly expressed in human cell line PER.C6. Pentamer and hexamer forms of this IgM were purified for full glycosylation profiling.

In IgG, differences in glycosylation profile and site occupancy at the single *N*-glycosylation site at Asn297 have been reported widely to implicate host immune response. IgM has 5 *N*-linked glycans on each heavy chain, with a total of 51 *N*-glycosylation sites on the pentamer and 60 *N*-glycosylation sites on the hexamer. The effects of glycosylation on IgM-mediated immune response have not been explored thoroughly.

Using LC-MS/MS, we show that glycopeptide analysis of both pentameric and hexameric forms of the IgM contain differences in glycosylation site occupancy and glycosylation profile, despite having identical amino acid sequences.

In conclusion, characterization of the full glycosylation profile of IgM provides a stepping stone towards understanding effects of glycosylation on IgM-mediated immune response.

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Proteomic Analysis and Profiling of Malaysian Upland rice

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In this era, proteomics is one of the omics research fields that are voraciously studied in order to understand more on gene expression and genome annotation. Rice has played a very prominent role over centuries as a staple food and nutrient provider consumed by more than half of world populations. Even though there are many studies being carried out for wetland rice protein identification, none has been reported for upland rice. This preliminary study aims to help establish the proteome profile of Malaysian upland rice in the hope that it would be beneficial in understanding the proteins with diverse functions and predicting function of unknown proteins. In this study protein from upland rice was extracted by using TCA/Acetone based method. The protein pattern was compared on 1D SDS-PAGE, the excised gel bands and total protein were digested with trypsin and analyzed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). The MS/MS spectra obtained were subjected to search against *Oryza sativa* database. Comparison of the protein on 1D SDS-PAGE showed similar pattern for all cultivars. However, analysis via mass spectrometry revealed that the same protein presented may not be detected in all cultivars. More than 200 proteins have been identified and most of the proteins found are responsible for the growth and development of the plant, stress tolerance, antioxidant and natural sweetener. This finding gives insightful information with regards to the various proteins expressed and synthesized in upland rice that could contribute to a better understanding of its physiological significance. It could bring us one step closer to determine the function of every gene in upland rice particularly the one that is closely related with its growth and development.

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ISD[n]: in-source decay and MS[n] of proteins with post-translational modifications using Matrix-Assisted Laser Desorption Ionization-Quadrupole Ion-Trap Time-of-Flight Mass Spectrometer

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MALDI-ISD is a useful technique for N- and C-terminal sequencing of intact proteins. MALDI is commonly classified as a *soft* ionisation technique where laser powers close to threshold values generate intact protonated peptide and protein Ions. In the case of peptides, increasing the laser fluence results in unimolecular decomposition in the time-of-flight region – *post-source decay* (PSD). However, in the case of proteins, fragmentation can occur in the MALDI plume prior to ion extraction from the ion source i.e. *in-source decay*. Typically, protein identification is achieved by subjecting the protein(s) of interest to enzymatic digestion followed by MS/MS sequencing of the resulting peptides in a *bottom-up* approach. In contrast, MALDI-ISD is referred to as a *top-down* approach as sequencing is performed on the intact protein, thereby eliminating the lengthy digestion step.

Further benefits of the *top-down* approach are found in the analysis of post-translational modifications (PTMs) as even labile PTMs (that may be lost in a *bottom-up* approach) are retained thereby facilitating localisation during sequencing. MALDI-ISD does however have a limitation in the lower mass region where ISD fragment ions begin to overlap with chemical noise and matrix-related ions. A solution to this issue is to perform *pseudo* MS^3 through MS/MS fragmentation of precursor ions created by ISD. However, using conventional MALDI-TOF-TOF instrumentation, this approach is limited to *pseudo* MS^3. In this application, we report ISD[n]: the extension of *top-down* sequencing to maximise sequence coverage of intact modified and unmodified proteins by using a unique MALDI-QIT-TOF for MALDI-ISD and *pseudo* MS^5.

The advantages of ISD[n] over a conventional ISD analysis of proteins are demonstrated using recombinant Tau protein. Tau has been reported to be involved in Alzheimer's disease and is a good *in vitro* model for protein modification analysis. The unmodified protein along with the corresponding oxidised form, were used to demonstrate the ability of the described technique for the characterisation of modified proteins.

The ISD[n] spectrum obtained for unmodified Tau contained both N-terminal cn- and bn-ions and C-terminal yn-ions. ISD[n] analysis of oxidised Tau did not show significant losses of sulfenic acid (64 Da) indicating the modification remained intact during analysis. In addition to N-terminal cn-ions, c-ions showing both a mass shift of +16 Da: oxidised Met10 (sulfoxide form) and +32 Da: oxidised Met10 (sulfone form) were also detected. C-terminal yn-ions and y-ions showing a mass shift of +16 Da: oxidation of Met418 were also observed.

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Moonlighting proteins of *Mycoplasma hyopneumoniae* and their role in pathogenesis

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Mycoplasma species are strictly parasitic bacteria that have undergone extensive genomic reduction and therefore biochemical efficiency is essential for their survival. They require their host for the essential supply of lipids, amino acids and nucleotides for growth. The pig pathogen *Mycoplasma hyopneumoniae* infects the respiratory system of pigs and causes significant agricultural losses. One hypothesis is to explain their success as chronic pathogens suggests that the Mollicutes have evolved multifunctional proteins as a means to increase protein function without expanding gene content.

Multifunctional proteins were first described in 1995 and have since been found in all biological kingdoms. Bacterial moonlighting proteins have been found to participate in key host-pathogen interactions and primarily function as adhesins, metabolic enzymes, ribosomal proteins, chaperones, and proteases. Moonlighting proteins can be detected in cellular fractions other than those stated by initial annotation and online databases, and by identifying the secondary functions of known proteins. The extracellular function of these proteins expressed by bacteria are predominantly roles in host interaction, primarily binding to extracellular matrix or host cell surface molecules, but also in modulating host immune function. Studies have shown that glycolytic enzymes which are conserved in both the pathogen and the host can introduce autoimmunity in the host organism.

Specific surface-exposed proteins from *M. hyopneumoniae* identified by cell-surface proteomic analyses were selected as possible moonlighting proteins because of their annotations and bioinformatics predictions to be cytosolic. The natively purified recombinant proteins were characterised for heparin, plasminogen and fibronectin binding using microscale thermophoresis, blotting techniques and heparin affinity chromatography to elucidate their functional role as moonlighting proteins. With the use of protein modelling programs and bioinformatics software, the host molecule binding motifs specific for heparin and therefore potential binding sites on these putative moonlighting proteins can be identified.

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Quantitative shotgun proteomics analysis of high and low antioxidant expressing Australian recombinant inbred lines of *Arachis hypogaea*.

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Peanuts are rich in essential nutrients, including proteins and antioxidants and are favoured by consumers seeking healthy food options. Peanut breeders are keen to develop cultivars with enhanced valuable functional food traits, in addition to disease resistance and high kernel yield potential. For example, peanut cultivars with high oleic acid content >75% have recently been developed by hybridisation using a naturally occurring mutant line developed by the University of Florida in the USA. In an effort to breed a stable peanut cultivar with enhanced antioxidant content, a population of recombinant inbred lines (RILs) was developed by the Australian Peanut breeding program through hybridisation by crossing a full season peanut genotype consistently expressing high antioxidant capacity (D147-p3-115) with another line consistently expressing low-mid level antioxidant capacity (Farnsfield).

A subset of RILs (p27-272, p27-363, p27-036) showing very high and very low total antioxidant capacities and parent lines (D147-p3-115 and Farnsfield) were selected for proteomics analysis. A label-free quantitative shotgun proteomics approach coupled with nano-LC MS/MS was adopted to study functional proteins altered in peanuts with different antioxidant capacities. Due to the lack of the complete peanut genome, peptides were identified using a customised peanut protein sequence database..Statistically significant differentially expressed proteins between peanut lines with high and low antioxidant capacities were identified, and mapped to biological pathways. Protein abundance changes in biological pathways reveal the molecular fluctuations mediating cellular processes underlying each of the different RILs. Potential protein biomarkers were identified for high antioxidant expressing peanut lines of *Arachis hypogaea.* These markers could help breeders in developing correlations between antioxidant responsive proteins and genes that account for the observed phenotype. This information could potentially enable breeders to integrate this with marker assisted selection for the breeding of high antioxidant cultivars.

Keywords: Peanuts, antioxidant capacity, Shotgun proteomics, nano LC-MS/MS, biomarker discovery.

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Identification of protein-protein interactions of PfRh5 required for the invasion of human red blood cells by malaria parasites

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Plasmodium falciparum causes the most severe form of malaria in humans causing over 700,000 deaths each year. Invasion of human erythrocytes is essential for its survival and this process involves the interaction of multiple host-parasite receptor-ligand interactions. One of the most important is that of the parasite ligand PfRh5 with the host receptor basigin. PfRh5 is the leading blood-stage vaccine candidate that elicits potent strain-transcending invasion inhibitory antibodies. We have determined the crystal structure of PfRh5 to a resolution of 2.18 Å (Chen *et al.*, PNAS 2014). The PfRh5 adopts a novel fold with the core structure consisting of seven α-helices winding around two β-sheets in an anti-parallel fashion to form a flat and compact molecule. Chemical cross-linking mass spectrometry and molecular modelling of the PfRh5-basigin interaction suggested that the receptor binds at one end of PfRh5, providing other areas for interaction with other proteins, such as PfRh5 interacting protein, PfRipr (RH5-interacting protein 1). Quantitative MS analyses of Co-IPs using HA-tagged PfRipr1 or pull-downs using

recombinant Rh5 as a bait demonstrated that PfRH5 and PfRipr 1 associates with a novel conserved protein (PfRipr2) to form a complex on the merozoite surface. This discovery elucidates a novel protein that may tether PfRH5 to the merozoite surface and facilitate its binding with basigin. Our insights open potential avenues for novel anti-malarial strategies that may inhibit the formation of the crucial basigin-PfRH5-PfRipr1/2 invasion complex.

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Characterization of Non-Competitive Imidazoline Inhibitors of the Human Proteasome by LC-MS/MS

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Inhibition of the ubiquitin-proteasome pathway using bortezomib has emerged as a rational strategy for the chemotherapeutic treatment of multiple myeloma (MM). However, the competitive inhibition mechanism of bortezomib[1] results in >97% of patients becoming resistant or intolerant to treatment in the first few years, after which survival is typically less than a year[2]. Non-competitive proteasome inhibitors, such as imidazoline scaffolds, that regulate the proteasome via a mechanistically distinct protein:ligand interaction, and that overcome resistance and are highly effective in vivo[3], have therefore received recent interest for overcoming the limitations of competitive inhibitors. However, little is known about the site(s) of non-competitive inhibitor binding to the proteasome, or how they exert their biological activity. Here, we have employed a strategy involving immunoprecipitation and quantitative LC-MS/MS analysis of ubiquitinylated substrates, to gain insights into the non-competitive human proteasome inhibition mechanism of imidazoline scaffolds TCH-013 and TCH-165, compared to the competitive inhibitor bortezomib. Cytosolic proteins extracted from RPMI cells incubated with 1.0 µM TCH-165, 10.0 µM TCH-013, 0.1 µM Bortezomib or 1.0 % DMSO for 1 hour were treated with ubiquitin antibody conjugated to agarose beads, then eluted with 0.1 M glycine at pH 2.5 and 4 °C for 10 min. Each protein sample was then digested using trypsin, Lys-C and Asp-N. Quantitative analysis of differential protein abundances between the different treatment groups was performed via dimethyl labelling, prior to LC-MS/MS using a Thermo Q-Exactive Plus mass spectrometer. Protein identification and quantitation was achieved using Mascot database search software. Preliminary results from this study indicate that the competitive proteasome inhibitor bortezomib presents vastly different and more abundant ubiquitinylated protein profiles compared to the non-competitive inhibitors, indicating a lack of accumulation of some proteins with non-competitive inhibition, and supporting the hypothesis that imidazoline scaffold inhibitors present a drug-induced substrate specific inhibition of proteasome mediated protein degradation.

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The secretome of *Wallemia ichthyophaga*.

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The subphylum *Wallemiomycetes* contains three species distinguished by both genetic and morphological characteristics: *W. ichthyophaga*, *W. sebi* and *W. muriae*[1]. These species have been isolated from environments with low water availability and are considered to be the most xerophilic and halophilic fungi isolated to date[2]. *W. ichthyophaga* requires at least 10% NaCl to grow and thrives in saturated salt media. We isolated a strain of *W. ichthyophaga* from salted sheep pelts, which were subsequently dewooled in water without any noticeable skin damage. We hypothesised that enzyme(s) secreted by the fungus might be facilitating the dewooling process, and so began to investigate the secretome of the sheep pelt isolate. We undertook a geLC-MSMS approach to identify the proteins secreted by the organism after two months growth in Wilson's media containing 20% NaCl. Clarified culture medium was concentrated and desalted by ultrafiltration. Six separate samples were analysed; three technical replicates of two biological replicates, and positive identifications were made only when stringent criteria were met. This resulted in the identification of only 22 unique secreted proteins which was less than expected, as during the secretome analysis, the genome of *Wallemia ichthyophaga* EXF-994 was published and is predicted to encode 152 "highly likely secreted" proteins. We therefore repeated the experiment without pre-fractionation (*i.e.* using LC-MSMS on the trypsinised desalted concentrated secretome), and these results will be reported. One of the identified glucosidases was further purified using IEC and SEC, and characterised. The enzyme was shown to be N-glycosylated, was most active at 10% NaCl, and interestingly, appeared to be able to hydrolyse both beta-(1,4) and alpha-(1,4) linkages.

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A Reliable and Repeatable sublimation based protocol for the analysis of Formalin Fixed Paraffin embedded (FFPE) tissue via Matrix Assisted Laser Desorption Ionisation Imaging Mass Spectrometry (MALDI-IMS)

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Matrix Assisted Laser Desorption Ionisation Imaging Mass Spectrometry (MALDI-IMS) is a robust technique that has seen great improvement in recent time and has the potential to further the field of diagnostic imaging. However with automated sample preparation approaches becoming more and more prevalent, little work is being done to understand and standardize the critical matrix application and co-crystallisation techniques in use, or investigate more economical non-machine based approaches. Our research has led us to dissect and define the conditions responsible for the successful implementation of a sublimation based approach for sample preparation. By quantifying each condition and empirically determining their ideal conditions, we propose a repeatable and cost effective method for the universal MS imaging of Formalin fixed paraffin embedded (FFPE) tissue. In this work, we have demonstrated a universal tissue preparation and matrix application technique with low startup costs that can be operated by "walk-up" users and provide explanation and background information for further customization and optimization by experienced and inexperienced operators alike. We as also describe a novel technique for a non-delocalising method of Trypsin application. The application of such a technique has also opened up new avenues of research that allow for the combination of disparate methodologies to incorporate IMS as a standard indicative technique; We are aiming for the integration of IMS as a standard operating protocol in the same way other techniques have become ubiquitous in the lab. Only through the dissemination of complete and unabridged protocols and the empirical determinations for the conditions that govern them, will integration of disparate techniques create workflows with global perspective and therefore global results.

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Comparisons and optimizations on the TripleTOF 6600 and Orbitrap platforms

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The choice of a specific mass spectrometer for a specific proteomic application is critical. Furthermore, the combination of optimized settings with efficient data analysis pipelines or parameters can heavily influence results. The aim of this study was to maximize protein identification on instruments available in the Mass Spectrometry Core Facility at the Sydney University. A single tryptic digest of mouse liver was analysed using highly similar chromatography conditions on a variety of instruments including the TripleTOF 6600, Q-Exactive and LTQ-Oribtrap Velos Pro. Initially, optimization of settings was performed on the TripleTOF 6600 to maximize protein identification with nanoUHPLC and long gradients. A "Fast" and "Sensitive" acquisition strategy was devised similar to (Kelstrup et. al., *J. Proteome Res.,* (2012), 11(6); 3467) and used to compare triplicate high load injections for single-shot proteome analysis. Next, data-analysis pipelines were directly compared including Proteome Discoverer, MaxQuant and Protein Pilot. These data will provide valuable insights into efficient protein identification with various platforms.

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Evaluation of a lipid profiling system using reverse-phase liquid chromatography coupled to high-resolution orbitrap mass spectrometry and automated lipid identification software

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A lipid is generally considered to be any molecule that is insoluble in water and soluble in organic solvents.

Biological lipids usually refer to a broad grouping of naturally occurring molecules which includes fatty acids, waxes, eicosanoids, monoglycerides, diglycerides, triglycerides, phospholipids, sphingolipids, sterols, terpenes, prenols, fat-soluble vitamins (such as vitamins A, D, E and K) and others.

The main biological functions of lipids include their central role in energy storage, as structural components of cell membranes, and as important signaling molecules

Mass spectrometry is used frequently for lipidomics, but is not currently in general use at UNSW. As a facility we frequently face problems such as inexperienced instrument users, poor sample preparation practices and a lack of understanding of the experiments involved.

Technical advances in instrumentation (UHPLC and Orbitrap mass analysers) and in software for qualitative and quantitative lipidomics have provided us with a potential 'turnkey' system with robust characteristics and production of high quality data. This type of approach cannot replace dedicated lipidomics laboratories and specialists, but it is hoped that it will provide a general lipidomics solution in a facility environment.

Initial results and evaluations of the system combining LipidSearch software with a Q-Exactive Plus mass spectrometer and C18 UHPLC are presented and discussed.

Results obtained from mouse liver extracts will be compared with those obtained from the same samples measured using the current gold standard - 'shotgun' direct infusion analysis on a QTrap instrument.

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Investigation of heat stress in cattle using multiple reaction monitoring (MRM) mass spectrometry to measure cytokine levels in plasma.

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Feedlotting cattle is a common strategy to increase growth rate and the quality of the meat from beef cattle. However, the combination of tropical high humidity and climatic temperatures with the carbohydrate and protein rich diet supplied to cattle in

this production system can result in deleterious effects on metabolism, heat stress and even heat stroke in extreme cases. Heat stress triggers an inflammatory process which in turn triggers the production of a group of key plasma cytokines: interleukin-1 beta (IL-1β); interleukin-6 (IL-6); interferon gamma (INF-γ) and tumor necrosis factor alpha (TNF-α). These plasma cytokines are found in low concentrations (0.03 to 0.3 pmol/μL) as measured by enzyme-linked immunosorbent assays (ELISA). In this research, we aimed to develop and optimize a mass spectrometry-based assay employing multiple reaction monitoring (MRM-MS) to detect and quantify plasma cytokines at low concentrations. For MRM-MS method development commercially available recombinant cytokines were digested with trypsin and analysed by high resolution MS/MS analysis to select peptides useful for quantification and for MRM design. Samples were chromatographically separated on a Shimadzu Nexera HPLC system (Shimadzu, Australia) using a Phenomenex Kinetex C18 (2.1 x 100 mm) column and a linear gradient of 5-45% acetonitrile over 6 min with a flow rate of 400 μL/min. The eluent from the HPLC was directly coupled to a 6500 QTRAP MS/MS system (AB/Sciex, Foster City, USA) equipped with a TurboV ionization source operated in positive ion mode. Three MRM transitions for each candidate peptide biomarker were selected based on MS response and the collision energy (CE) for each transition was optimised. Using five peptides per cytokine the dynamic range of detection was assessed in aqueous solution. Cytokine extraction will be assessed and optimised using established protocols including solid-phase extraction (SPE) or acetonitrile precipitation. Method development and optimisation experiments have demonstrated that MRM-MS is a sensitive and selective alternative to ELISA capable of identifying and quantifying cytokines at concentrations below 2 nmol/μL.

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Proteomic exploration of cyclic peptide diversity among plants using peptide labelling and orthogonal separation strategies

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Plants have evolved various defense strategies to counter the constant threat from predators and infectious organisms. Aside from physical deterrents such as thorns, plants produce a chemical arsenal consisting of small molecules typically assembled from isoprene units, alongside a range of defense peptides. Cyclotides, found in a range of plant species are ribosomally synthesised defense peptides defined by their unique combination of a cyclic peptide backbone and cystine-knotted topology.[1] Challenges faced in the proteomic analysis of cyclotides stem from difficulties in their chromatographic separation, the necessity for linearisation prior to MS analysis, and their typically poor fragmentation. Here we examine the benefits of orthogonal separation and chemical derivatisation prior to tandem MS in the proteomic characterisation of cyclotides.

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IDENTIFICATION OF SEED PROTEOME OF MALAYSIAN UPLAND RICE

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Rice (*Oryza sativa* L.) is immensely consumed by global population and becoming the staple food in many countries. Providing a lot of nutrient content, rice comes in various variety, shapes, color and taste. Typical rice breeding is in flooded condition with good irrigation system referred to as wetland variety hence, requiring substantial amount of land. Our focus is on varieties that can sustain drought condition and require minimal amount of water for its growth known as upland rice. Upland rice in Malaysia is mostly grown either on hilly side or low land area without subjecting the plants to flooded condition where the source of water comes from rainfall. We set out to analyze a few of Malaysian upland rice varieties to identify the proteins to establish seed proteome database for rice from Malaysia. Protein were extracted from the seeds using suitable method adapted from Singh et al (2004) followed by separation on 1D SDS-PAGE, tryptic digestion and peptide sequencing by LC-MS/MS. Identified proteins were then searched against *Oryza sativa* databases. We confirmed the presence of water stress inducible protein which confers the upland variety tolerance to drought aside from many other proteins such as storage proteins, embryo-specific proteins, stress-related proteins, signaling proteins and several antioxidants. Responses to stress, biotic and abiotic stress, and protein metabolism are the largest representation of GO analysis. Our findings presents a preliminary insight into the seed proteome of Malaysian upland rice which could provide an alternative to typical rice breeding due to land scarcity.

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Development of an 'Immuno-LC-MS/MS' Assay for the Quantitative Characterization of Oxidized and Truncated Parathyroid Hormone (PTH): Implications for the Treatment of Patients with Chronic Kidney Disease

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Parathyroid hormone (PTH) plays a critical role in the regulation of circulating blood calcium levels, and serves as a biomarker of secondary hyperparathyroidism associated with the diagnosis of disorders such as vitamin D deficiency or chronic kidney disease (CKD), and for monitoring the effectiveness of treatment e.g., hemodialysis. Immunochemiluminescent assay of full length (i.e., 1-84) PTH has historically been problematic due to variable standardization, cross reactivity with truncated PTH fragments (e.g., 7-84), and variable correlations with biological parameters. A recent report that PTH may be oxidized *in vivo* further complicates these issues, since oxidized PTH is biologically inactive.

To address these challenges, an immunocapture liquid chromatography–tandem mass spectrometry ('immuno-LC-MS/MS') analysis strategy was developed and optimized, together with a novel dual isotope-labeled internal standard approach (i.e., ^{15}N-labelled full length and truncated PTH and $^{13}C_6{}^{15}N$ isotope-labelled oxidized PTH tryptic peptides), in order to (i) quantitatively determine the nature of circulating full length and truncated PTH and their individual oxidized isoforms, (ii) differentiate between *in vivo* and *ex vivo* oxidative modifications, and (iii) examine the effects of PTH oxidation on current immunochemiluminescent PTH assays. First, using a series of purified full length, truncated and individual oxidized isoforms of PTH spiked into depleted plasma, the recovery of the overall analysis strategy was determined to be 66%, with a wide linear range for detection from 5-2000 pg/mL.

Then, two series of plasma samples from patients on hemodialysis were analyzed. In Set A, oxidized full length and truncated PTH were commonly observed, with oxidation ranging from 0 to 80% of the total PTH observed, and with 34 of the 41 samples containing more than 10% oxidization. In general, there was more oxidized PTH 7-84 than oxidized PTH 1-84. In contrast, in Set B, only minimal amounts of oxidized PTH peptides were observed, with only 3 of 29 samples containing >10% oxidation. The marked difference in oxidized PTH peptides between Set A and Set B suggests that oxidation of PTH may be occurring *ex-vivo*. Further studies are required to fully define the possibility and origin of *ex-vivo* oxidation. The immuno LC-MS/MS results correlated extremely well with the assay of PTH 1-84 and less well with other "intact PTH" assays. The effects of oxidation on the results obtained using current intact PTH assays may represent an additional factor contributing to the variability of PTH assay results and their correlation with biological parameters.

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Revisiting the arthritogenic peptide theory: Quantitative - not qualitative - changes in the peptide repertoire of HLA-B27 allotypes

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The association of Human Leukocyte Antigen (HLA) B27 with spondyloarthropathy is one of the strongest documented for any autoimmune disease. A common hypothesis for this association is the arthritogenic peptide theory. It dictates that differences in the peptide binding preferences of disease-associated and non-associated HLA-B27 allotypes underpin the presentation of bacterial and self-peptides leading to cross-reactive T cell immunity and subsequent autoimmune attack of affected tissues.

Using high-resolution mass spectrometry in combination with SWATH-MS and MRM, we have analyzed and comprehensively quantified the peptide repertoires derived from the 8 most common HLA B27 allotypes, HLA-B*27:02 to HLA-B*27:09. Differences in the peptide binding pockets of these molecules manifest largely as quantitative, but not qualitative changes in the peptide cargo. Thus, absolute binding preferences do not explain disease-association and the arthritogenic peptide theory needs to be reassessed in terms of quantitative changes in self-peptide presentation, T cell selection and altered conformation of bound peptides.

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Detection of novel beta-catenin protein interactors in colon cancer

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Beta-catenin is a protein associated with several human cancers. The progression of cancer is correlated with nuclear accumulation of beta-catenin, a process which is regulated by phosphorylation. Protein phosphorylation also alters function through changing protein-protein interactions. In the context of colon cancer we are interested in furthering our understanding of these two aspects of beta-catenin regulation.

We generated recombinant human full-length beta-catenin constructs to achieve phospho-mimic and phospho-dead Y654 in the Arm 10-12 domain. These constructs were used along with wildtype beta-catenin to enrich for protein binding-partners in SILAC labelled SW480 and HT29 colon cancer cell lines in triplicate.

Eluates were recovered, separated by SDS-PAGE and analysed by mass spectrometry (Orbitrap ELITE). Proteome Discoverer 1.3 was used for data analysis and candidates selected based on presence in all replicates at least 2-fold more than the control. Detection of at least 2 peptides and replicates having a p-value <0.05 (one-sided t-test) was also applied.

For SW480, candidates include 225 for the wildtype, 49 for the phospho-mimic and 62 for the phospho-dead. For HT29, candidates include 66 for the wildtype, 146 for the phospho-mimic and 22 for the phospho-dead. Several well-known beta-catenin interactors were detected including catenin alpha-1, catenin delta-1, adenomatous polyposis coli protein (APC), four and a half LIM domains protein 2, ruvB-like 1, and paxillin.

Novel candidate interactors were grouped into processes and functions including nuclear import and export, regulation of cell cycle and mitochondrial proteins. We selected 3 candidate proteins (OPA1, Cdk1, Hsp70) as potential novel interactors and attempted to validate by immunofluorescence microscopy and an in situ proximity ligation assay (DuoLink). This presentation will report our progress towards confirming these proteins as novel beta-catenin effectors.

Zinc-α-2-glycoprotein (ZAG) is over expressed in the milk of a Pteropid bat (Pteropus alecto)

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Bats have been identified as reservoirs for a number of zoonotic viruses, including Ebola virus, Henipah viruses, SARS-like coronavirus and Australian bat lyssavirus. Bats have the ability to transmit these viruses (a process known as spillover) to other susceptible species leading to severe illness and in some cases death. While these bat borne viruses may cause significant disease in their spillover host, bats remain asymptomatic and rarely show any clinical signs of disease. The molecular mechanism by which bats control viral infection is currently unknown and is therefore of significant value.

Mammalian breast milk is rich in nutrients, hormones, growth factors and immunoactive molecules, which influence the growth, development and immune status of a newborn infant. It is hypothesized that the nutritional function of milk evolved subsequent to its protective function; that is, the mammary gland evolved from the innate immune system.

The comprehensive proteomic analysis of Pteropid bat milk, presented in this study, revealed that it is very concentrated and rich in proteins, with the majority of typical milk proteins identified. The most distinctive and interesting feature of *P.alecto* milk was the over production of zinc-α-2-glycoprotein (ZAG). This protein was detectable, albeit in much lower level, in human milk but was not detected in milk of other mammals analysed, including an Australian microbat (*Chalinolobus gouldii*).

ZAG is a protein of interest due to its postulated multifunctional role, although it is still considered a protein with an unknown function. One role postulated is as a novel adipokine (lipid metabolism regulatory molecule), whereas another proposed role is, due to structural organization and fold similar to MHC class I antigen-presenting molecule, in the expression of the immune response. Hence the overexpression of ZAG in bat milk may be related to the molecular mechanism by which bats control viral infection.

Irreversible Oxidative Modification of Cysteines Resulting from a Prolonged High Fat Diet Model

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The extent of oxidative stress on the myocardial molecular environment due to obesity is yet to be fully understood. The cellular response to increased oxidative stress is regulated in part, by highly reactive thiol groups on cysteine residues undergoing various oxidative modifications that regulate protein function. Pathologies such as obesity lead to the formation of aberrant oxidative modifications on proteins, such as the largely irreversible sulfinic and sulfonic acids. This study aims to utilise a novel enrichment strategy to identify proteins that undergo these oxidative modifications as a result of obesity. Rats were fed a CHOW (12% fat) or high fat (HF) (42% fat) diet for 6 months. To quantify alterations in protein abundance, samples were isobarically tagged prior to mass spectrometry (MS) analysis. In parallel, irreversibly oxidised peptides were negatively selected utilising low pH loading conditions for strong cation exchange (SCX), whereby the negative charge imparted by the over-oxidation of Cys limits interaction with the chromatography. The resulting SCX fractions were further partitioned by hydrophilic interaction chromatography prior to MS analysis. Irreversibly oxidised Cys peptides were increased 2.5 fold in the HF diet, with 10 unique irreversibly oxidised Cys-containing peptides observed in the CHOW diet (10 unique proteins) compared with 22 observed in the HF diet (arising from 20 unique proteins). 55 discrete proteins were observed to be significantly changing in abundance as a result of the HF diet. These proteins were largely found to be associated with fatty acid metabolism, degradation and oxidation. The most prevalent of these proteins identified as oxidised due to the HF diet was β-actin. This irreversible oxidation may cause stiffening of the cytoskeleton, impacting the arrangement and functionality of the z-disk, as shown in hypertrophic models. Given that hypertrophy is a comorbidity associated with obesity, we suggest that this alteration to beta-actin has functional implications in this model. This study is the first application of this novel method of enrichment for irreversibly oxidised Cys proteins to obesity, identifying key proteins associated with important functional groups. Understanding these modifications and their impact on myocardial function may provide novel mechanisms of treatment to improve recovery from AMI in the high-risk obese population.

Identification of apoE and amyloid beta complexes in human plasma.

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The cholesterol and lipid transporter, apolipoprotein E (ApoE), has an important although uncertain role in the pathogenesis of sporadic Alzheimer's disease (AD). ApoE's importance is demonstrated by the significantly increased risk of developing alzheimer's disease between the three main alleles of APOE, ε2, ε3, ε4. ApoEε4 is the most jeopardizing allele, conferring a dose-dependent increase to the risk for AD and reducing the mean age of onset from 84 to 68 years old in homozygous ε4

persons, compared to persons with no ε4. Whereas ε4 is second only to age in importance amongst AD risk factors, its presence is neither necessary nor sufficient to cause AD, suggesting that environmental or other genetic elements contribute to the disease. Here we show using large-format, narrow pI range (4.7-5.9), 2D-PAGE proteomics of heparin-sepharose binding proteins from homozygous (ε2/ε2, ε3/ε3, ε4/ε4) human sera. We find no differences in total spot volumes from ApoE spot trains between heparin-Sepharose enriched sera from healthy or Alzheimer's disease ApoE carriers. Furthermore, there were no significant differences found between ε2, ε3 or ε4 carriers of native ApoE migrating at 34 kDa. However, a reduction-resistant variant migrating at 45 and 55 kDa were found in ε3/ε3 and e2/e2 individuals respectively. Mass spectrometry identified these species to be complexes of ApoE, ApoAII and amyloid β. These data suggest that ApoE isoforms have unique functions that require further investigation.

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Dissecting protein dynamics involved in reactive oxygen species-induced insulin resistance in adipocytes using SILAC-based quantitative proteomics

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The production and accumulation of reactive oxygen species (ROS) may contribute to the development of insulin resistance, which is the vital feature of type 2 diabetes. Thioredoxin and glutathione are the major thiol antioxidants protecting cells from ROS-induced cytotoxicity. In this study, carmustine (BCNU) was used to specifically inhibit glutathione reductase and auranofin (AF) was used to specifically inhibit thioredoxin reductase. In assay1, BCNU was used to stimulate 3T3L1 adipocytes for 2h and 24h. Glucose uptake was significantly inhibited by 2h-long stimulation, but not affected by 24h-long stimulation. In assay2, BCNU and AF were used together to stimulate 3T3L1 adipocytes for 2h and 24h. Glucose uptake was dramatically inhibited by both 2h- and 24h-long stimulation. Using SILAC-based quantitative proteomics, we quantified 4533 and 4111 proteins respectively. Based on fuzzy c-means analysis, these proteins were clustered into eight groups, and some of them were tightly associated with glucose uptake changes. In BCNU-stimulation assay, proteins with negative glucose uptake correlation were mainly located in mitochrodria, enriched in oxidative phosphorylation pathway. However, proteins with positive glucose uptake correlation were mainly enriched in Proteasome. In BCNU/AF-stimulation assay, proteins with both negative and positive glucose uptake correlation were mainly located in mitochrodria. The former were enriched in oxidative phosphorylation pathway, but the latter played key roles in generation of precursor metabolites and energy, enriched in glycolysis/gluconeogenesis pathway and some other metabolism-related pathways. Several dynamic key proteins and well-known phosphorylation sites perhaps involved in insulin resistance were validated by western blot.

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Moonlighting with Melanoma and the Unfolded Protein Response

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Introduction and Objectives

Metastatic melanoma is a lethal neoplasm with rapid progression and systemic dissemination. The 5-year survival rate for patients with distant metastases is just 6%-18%. This is due not only to the aggressive nature of the disease but also a lack of effective therapies. Activation of the unfolded protein response (UPR), a stress response, in melanoma positively correlates with tumour progression, metastasis and poor outcome. The complete action of the UPR is yet to be determined, and its role in cancer progression is undefined.

Method

In the present study, proteomic analyses have been used to identify proteins involved in the UPR and to elucidate its role in melanoma. Subcellular proteomes of human melanoma cell lines (Mel-RM and WMM1175) treated with thapsigargin (TH), an inducer of ER stress, were labeled with iTRAQ and anaylsed using 2D LC-MS/MS. Differentially abundant proteins were validated using selected reaction monitoring (SRM) mass spectrometry and western blotting. Membrane-associated progesterone receptor component 1 (PGRMC1) and 4F2-cell surface antigen were labeled with Cy5-conjugated antibody and the sub-cellular trafficking with TH treatment monitored by fluorescence microscopy. Finally pull-downs of PGRMC1 and 4F2 were performed to elucidate their role in the UPR.

Results and Discussion

Following activation of the UPR, 80 differentially abundant proteins were identified by mass spectrometry. These proteins are involved in stress response, cell migration and adhesion, and apoptosis/survival. PGRMC1 and 4F2 are up-regulated and translocated as a result of UPR activation, both proteins have been linked with cancer progression and their association with the UPR novel.

Conclusions

The UPR affects several important oncogenic proteins and pathways, such as the Akt and MAPK pathways, showing the importance of the UPR in melanoma progression. The differentially abundant proteins identified here extend our understanding of how the UPR can modulate protein function and contribute to melanoma tumourigenesis.

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Post-translational processing, multifunctionality and moonlighting: The not-so-simple proteome of a genome-reduced pathogen

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Mycoplasma hyopneumoniae is an economically significant pathogenic bacterium that chronically infects the respiratory tract of swine. This infection leads to pneumonia and secondary infections, costing agricultural industries significantly in the use of antibiotics and vaccines, which are currently largely ineffective. An improved understanding of the molecular mechanisms behind the infection process is essential to our ability to rationally design better vaccine and therapeutic interventions. With fewer than 700 predicted protein coding sequences, *M. hyopneumoniae* possesses one of the smallest genomes of any free-living organism. As such, it lends itself well to thorough proteomic interrogation.

A slew of proteomic techniques have been used to investigate the global proteome and surface-proteome at the protein and peptide level, including surface shaving and labelling techniques, ligand and immuno-blotting and affinity chromatography, as well as N-terminal dimethyl labelling to determine true N-termini of mature proteins.

This conceptually unbiased, function-oriented approach has revealed an unexpected level of complexity in the use of proteolytic processing, multifunctional proteins and moonlighting to compensate for reduced coding capacity at the genome level. While transcriptome studies suggest that the majority of genes are transcribed, our analyses identified less than 400 detectable expressed protein products. However, a significant number of these were discovered to be post-translationally modified, multifunctional or present at the cell surface, despite not possessing traditional signal peptides. Many of these proteins, predominantly cell surface adhesins, we have already described in the literature, however a surprising number of cytoplasmic "housekeeping" proteins are also found to be post-translationally cleaved, multifunctional or moonlighters. These findings can be applied to the development of vaccines and therapeutics in Mycoplasma, as well as having wider implications for the field of biology, if this level of post-translational regulation can be found in other bacterial pathogens.

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Identification of disease resistance genes in Sydney rock oysters using quantitative label free shotgun proteomics.

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The Oyster industry in Australia is one of the biggest contributing industries to Aquaculture. Sydney rock oyster (SRO) industry in particular has been severely affected by Queensland Unknown (QX) disease outbreaks, which is caused by a single celled protozoan, *Marteilia sydneyi.* Continuous outbreaks of this disease pose great risk for the sustenance of the industry, and hence development of disease resistant lines holds the key for the future of the industry. In 1990s NSW Department of Primary Industries started a selective breeding programme to develop a line of QX resistant SRO by performing interbreeding amongst the survivors of the QX outbreak. These oysters showed good disease resistance, but very little is known about the molecular mechanisms involved.

Previous studies have revealed that genes for disease resistance are heritable, so molecular level knowledge, will be a great help in the development of disease resistant lines. Using Marker assisted selection. We have performed proteomic comparison of QX resistant selected lines with Wild type oysters, using both 2D electrophoresis and label free shotgun proteomics. To get an insight into disease progression, we analysed the temporal proteomic variation of n=20 oysters of each type, collected from Hawkesbury River at two different time points during the disease progression. In an initial study using 2D gel comparison of QX resistant lines and wild type oysters, we found 38 proteins to be significantly (p<0.05) altered in expression in the disease resistant oysters. Quantitative label free shotgun proteomics will also be performed on the same protein samples to substantiate the findings further.

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Comprehensive Analysis of Recombinant Human Erythropoietin Glycoforms by Capillary Electrophoresis and Nanoflow Liquid Chromatography Coupled with Middle-Down Mass Spectrometry

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Introduction

Erythropoietin (EPO) is an essential glycoprotein for hormone red cell production. Recombinant human Erythropoietin (hrEPO) is used for treatment of anemia resulting from chronic kidney disease. EPO has three N-glycosylation sites and one O-glycosylation site which leads to high heterogeneity, making glycoform profiling challenging. Multiple formulations of hrEPO have been studied extensively by different approaches, including capillary electrophoresis (CE) and nanoflow liquid chromatography (nLC) coupled to advanced mass spectrometry (MS) analysis. Although CE-MS has been used to characterize intact EPO

glycoforms, quantitative mapping of each glycosylation site by CE-MS has not been previously accomplished. Here, we report that sheathless CE- and nLC-based separations coupled to Orbitrap middle-down MS provide complementary information and allow for comprehensive site-specific glycan mapping of rhEPO.

Methods

Reduced and alkylated rhEPO (Erythropoietin-Alpha, ProSpec, NJ) was digested with LysC (Roche, CA). Digested rhEPO **was analyzed on an LTQ Orbitrap Elite using CESI-MS or LC/MS coupled with high resolution high mass accuracy (HRAM) FT scanning of ETD or HCD MS2 fragment ions. Low flow sheathless CESI (Beckman Coulter, CA) employed both neutral coated and bare fused silica capillaries with integrated porous tip ESI sprayers. ProsightPC 3.0, Protein Deconvolution 3.0,** Proteome Discoverer 2.0 (Thermo Fisher Scientific) and Byonic (ProteinMetrics, CA) software packages were used for data analysis. A custom EPO glycan database was generated using proteinase K (Roche) digest and WAX RP-nLC-MS/MS analysis on Orbitrap Fusion. SimGlycan (PremierBiosoft, CA) was used for glycan identification.

Preliminary results

To generate the EPO glycan database, we used proteinase K to preserve site localization information. Short proteinase K glycopeptides were separated by a novel mixmode WAX-RP column. (Thermo Fisher), detected by HCDpdCID analysis on an Orbitrap Fusion and identified by SimGlycan. Identified glycans were used to compile the custom glycan database for glycopeptides identified by Byonic or Prosight PC.

To perform complete quantitative glycan site-specific mapping of rhEPO, we employed limited Lys-C digest to yield one glycosylation site per peptide fragment of 3-10 kDa size for middle-down analysis. This enabled more complete sequencing of glycoforms compared with top-down analysis. The rhEPO glycopeptides were well separated from non-glycosylated peptides by CESI and resolved over 20 min in a 50 min long run. Glycoform resolution was mostly based on differences in the number of sialic acid residues. The two predominant O-glycosylated peptides (N-acetylhexosamine-hexose with one or two sialic acids) migrated as completely resolved peaks. Due to high efficiency separation, we identified 14 glycoforms on Ser[126] by CESI-MS but only 4 by LC-MS using a Magic C18 AQ nano-LC column. Although our method demonstrated high efficiency separation and detection sensitivity of glycopeptides, we were initially unable to identify double N-glycosylated peptides containing Asn[24] and Asn[38]. Ultimately, we determined our EPO sample protein sequence was different from the one provided by the manufacturer. Using HRAM ETD and HCD fragmentation in combination with manual *de novo* sequencing and wild card searches (Byonic), we were able to identify the actual sequence of the EPO standard and N-linked glycopeptides. These results suggested that CESI-HRAM middle-down is a sensitive and efficient method for characterization of proteins or biopharmaceuticals with multiple glycosylated endogenous proteoforms.

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Reverse-Polynomial Dilution Calibration Methodology extends Lower Limit of Quantification and Reduces Error in Targeted Peptide Measurements in Blood Plasma.

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Matrix effect is the alteration of an analyte's concentration-signal response due to co-existing ion components. It is one of the most common problems leading to inaccuracy and imprecision in quantitative Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). With Electrospray Ionization (ESI) as the ion source, matrix effects are believed to be a function of the relative concentrations, ionization efficiency, and solvation energies of the analytes within the ESI droplet. For biological matrices such as plasma (with ~10^7 unique proteins when unfractionated and non-depleted), the interactions between droplet components is immensely complex and the subsequent effect on analyte signal response not well elucidated. This study comprised of three sequential quantitative analyses: We investigated whether there is a generalizable correlation between the range of unique ions in a sample matrix (complexity), the amount of matrix components (concentration), and matrix effect, by comparing an *E.coli* peptide-digest matrix (an approximate 2600 protein proteome) with phospholipid depleted human blood plasma, and unfractionated, non-depleted human plasma matrices (~10^7 proteome) for six human plasma peptide Multiple Reaction Monitoring (MRM) assays. Our dataset demonstrated significant analyte-specific interactions with matrix complexity and concentration properties resulting in significant ion suppression for all peptides ($p<0.01$), with 0>non-uniform effects on the ion signals of the analytes and their stable-isotope analogs. These matrix effects were then assessed for translation into error and precision affects in a low concentration (~0-250ng/mL) range across no-matrix, complex matrix (*E.coli* and phospholipid depleted plasma), and highly complex matrix (non-depleted plasma), when a standard addition Stable Isotope Dilution (SID) calibration method was used. Back-calculated error (%) and precision (CV%) across all matrices by SID were within <20%, however error in phospholipid depleted 20>20>and non-depleted plasma matrices were significantly higher compared to no-matrix and *E.coli* matrices ($p=0.007$). Finally a novel Reverse-Polynomial Dilution (RPD) calibration method with and without phospholipid-depletion was compared to SID for relative error and precision in a ~0-250ng/mL range in plasma. RPD techniques extend the Lower Limit of Quantification and reduces error ($p=0.005$) in low-concentration plasma peptide assays and is broadly applicable for verification phase Tier 2 multiplexed MRM assay development within the FDA-National Cancer Institute (NCI) biomarker development pipeline.

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Use of in vivo quantitative proteomics for the screening of candidate myokines

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Since the characterisation of Interleukin-6 as a myokine, the concept that skeletal muscle is a bona fide endocrine organ has gained considerable support. Here, we adopted the use of fully labelled ^{13}C Lysine SILAC mice to screen skeletal muscle for myokine candidates. We pooled the gastrocnemius muscles from three, unlabelled C57/BL6 mice either exposed to 90 minute treadmill running or non-exercised controls and combined 1:1 with a spike-in SILAC gastrocnemius standard. After separation of 10 fractions by SDS-PAGE and trypsin/Lys-C enzymatic digestion, the resultant peptides were analysed by LC-MS/MS using an Orbitrap Elite. In all, we identified 2280 proteins with a 5% peptide false discovery rate. 593 of these proteins were quantified across exercise and control experiments, 337 of which contained at least two SILAC ratios. Enrichment of the data set for the gene ontology cellular component, extracellular region or space, distinguished a selection of candidate myokines such as Decorin, Annexin A2 and Mimecan that are currently undergoing validation.

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The accessory protein, HMW1, of *Mycoplasma pneumoniae* is a multifunctional protein

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Mycoplasma pneumoniae (Mpn) is a major cause of community acquired pneumonia predominantly in children and the elderly. Mpn is remarkable in that it produces an attachment organelle that dictates how Mpn adheres to the human respiratory epithelium and the direction of motility. The attachment organelle focuses several key adhesion molecules to the tip of the organelle including the P1 and P30 adhesins. Three accessory proteins known as High Molecular Weight proteins HMW1, HMW2 and HMW3 as well as the product of the *mpn142* gene are thought to play a significant role in localising the adhesins to the attachment organelle. At present there is no data to suggest that these accessory proteins have a direct role in adherence and their cellular location is still unresolved.

In this study we characterised the function of HMW1. HMW1 is 112 kDa protein that migrates abnormally during SDS-PAGE at approximately 250 kDa. Four recombinant fragments spanning HMW1 were constructed as polyhistidine fusion proteins, expressed in *Escherichia coli* and purified using nickel-affinity chromatography. Rabbit antisera were raised to each to these purified fragments and used to localise HMW1 in the attachment organelle by immunofluorescence microscopy. In preliminary experiments aimed at determining if HMW1 plays a role in binding host molecules, affinity chromatography columns separately loaded with feutin, actin, fibronectin, heparin or plasminogen were constructed. Binding interactions between these bait proteins and native Mpn proteins were identified by LC-MS/MS. Our data suggests that HMW1 contains binding domains for several of these host molecules. Specific binding affinity involving different regions of HMW1 and host molecules will be measured by thermophoresis.

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Feature Extraction in MALDI Imaging using DIPPS

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MALDI Imaging is a promising new technology that allows for spatial heterogeneity in samples to be addressed. Signal intensity can be highly variable in MALDI Imaging experiments and adjusting for this variability can be difficult. We bypass the issue of signal intensity by considering presence/ absence data. This simplification allows proportions of occurrence, i.e. proportions of spectra with a peak at a particular m/z value, to be calculated. Using these proportions, signals that differentiate known spatial regions of interest can be identified by difference in proportion of occurrence statistics, or DIPPS. In many situations, this is ideal as it identifies a set of signals of interest that can serve as starting points for further analyses. We provide two example applications. The first example relates to an experiment designed to test if it is possible to detect N-linked glycans using MALDI Imaging by *in situ* application of PNGase. A control region of tissue was included, that was not treated with PNGase, and potential glycan signals are identified by occurring in the PNGase treated region but not the control region. The second example is an application to ovarian cancer tissue, where the interest is to identify tumour-specific signals. The tumour region is not annotated to begin with, and so is first separated by k-means clustering. Further analyses, including MS/MS identification, have been used to validate selected results in both example applications.

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Lysine Methylation Modulates the Protein-Protein Interactions of Yeast Cytochrome c Cyc1p

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In recent years, protein methylation has been established as a major intracellular post translational modification (PTM). It has also been proposed to modulate protein-protein interactions (PPIs) in the interactome. To investigate the effect of PTMs on PPIs, we recently developed the conditional two hybrid (C2H) system. It allows the co-expression of a modifying enzyme such as a methyltransferase, acetyltransferase, or kinase alongside the bait and prey proteins. Any increase or decrease in interaction due to the modification of the proteins can be measured by an increased or decreased level of reporter gene expression. With this, we demonstrated that arginine methylation can modulate PPIs in the yeast interactome. Here, we used the C2H system to investigate the effect of lysine methylation. Specifically, we asked whether Ctm1p-mediated trimethylation of yeast cytochrome c Cyc1p, on lysine 78, modulates its interactions with Erv1p, Ccp1p, Cyc2p and Cyc3p. We show that the interactions between Cyc1p and Erv1p, and between Cyc1p and Cyc3p, are significantly increased upon trimethylation of lysine 78. This increase of interaction helps explain the reported facilitation of Cyc1p import into the mitochondrial intermembrane

space upon methylation. This is one of the first demonstrations in a two-hybrid system that lysine methylation can modulate PPIs. It will be of interest to understand how widespread this process is in the eukaryotic cell.

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Antigen Presentation during Influenza Virus Infection

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CD8^{+} cytotoxic T cell (CTL) mediated immunity plays an important role in controlling and eradicating influenza virus in the host. CTL recognize major histocompatibility complex class I molecules (MHCI) in complex with viral peptides that are presented on the surface of antigen-presenting cells (APCs). T cell immunity directed against conserved CTL epitopes from internal influenza proteins provides an alternative vaccination strategy to current whole/split virus or subunit vaccines. However, the limitation in the development of suitable peptide-based vaccines is incomplete information on viral peptide processing and presentation and the impact of antigen presentation on the specificity of T cell responses. In this study we have quantified viral epitopes presented on the surface of H1N1-infected mouse dendritic and lung epithelial cells through a combined QTOF and triple quadrupole mass spectrometry approach. In total, 21 viral peptides were identified including two known immunodominant epitopes ($NP_{366-374}$ and $PA_{224-233}$), 10 known subdominant epitopes and 8 novel peptides. One of these novel epitopes was uniquely presented on the surface of infected epithelial cells. Compared to dendritic cells, lung epithelial cells were generally less efficient at peptide presentation: 2 peptides were not expressed at all, whilst 14 epitopes showed a level of expression less than half of that observed on dendritic cells. Across both cell lines, $NP_{366-374}$ abundance was the highest (over a thousand copies per cell), whereas $PA_{224-233}$ abundance was the lowest (~10 copies per cell), despite the co-dominant response to these NP and PA epitopes during primary infection in mice. These results indicate a disparity in the absolute levels of peptide presentation by different cell types, and their potential to affect T cell responses. The disconnect between epitope abundance and immunodominance provides an important insight into how T cells respond to infection. Moreover, the newly identified candidate viral peptides provide potential targets for the development of peptide-based vaccines.

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PROTEOMIC RESPONSE OF TWO DIFFERENT VARIETIES OF RICE EXPOSED TO DROUGHT STRESS AND RECOVERY

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Rice is the major staple food for more than half of world's population. As global climate changes, many places have seen changes in rainfall, resulting in more floods, droughts and severe heat waves. Cereal crops must be grown in hotter, drier and more hostile climates as expansion of the range of food crops accelerates, and climate change makes existing cropping zones more unreliable. Two rice varieties with contrasting genetic backgrounds, Nipponbare and IAC 1131, were used in this study. Nipponbare, the first fully sequenced agricultural plant, is a temperate-climate rice from Japan while IAC 1131, which is endemic to the hotter climate of upland rice fields in Java, is a drought tolerant upland tropical rice from the same sub-species as Nipponbare. We have grown both varieties and subjected them to drought stress and subsequent recovery, and collected material at each timepoint. Vegetative growth is cirtical for high productivity crops. We have focused on cell division – the variable for growth of any organism. The very small cohorts of dividing cells in the plant meristem have been collected via micro dissection and analyzed separately. We contrast this with protein expression patterns in the mature leaf blades, using both label free quantitative shotgun proteomic analysis and Tandem Mass Tags labelling. Preliminary results indicate 275 drought response proteins in Nipponbare while in IAC 1131 205 drought response proteins are identified. Cell biology analysis reveals that cell division in IAC 1131 is hardly affected by drought stress, however, the number of dividing cells in Nipponbare decreases significantly. The wealth of detailed information produced in this study will provides insight into the key proteins involved in leaf growth and drought tolerance.

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Evaluation of SWATH-MS for proteome profile quantitative analysis

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MS/MS[ALL] with SWATH acquisition is a data-independent acquisition strategy that generates high resolution MS/MS spectra for all detectable analytes in a biological sample within a pre-defined mass range. This technique enables high specificity MRM-like quantitation by extracting high resolution product ions of target analytes.

To evaluate SWATH as a proteome profile differential analysis technique, we acquired two sets of SWATH-MS data using two TripleTOF 5600 mass spectrometers for samples of human K562 whole cell protein digest spiked with 0.1%, 0.5%, 2%, 5% and 10% yeast protein digest. We extracted SWATH data using single IDA run library, comprehensive sample and instrument specific library, comprehensive sample specific library from other instrument, as well as publicly available generic libraries. We tested the effect of using different libraries to the quantitation results and determined the cut-off thresholds which would generate most true positives and most true negatives with least false positives reports for differential expressed protein discovery. The quantitation accuracy was also examined.

Our result demonstrated that it is feasible to extract SWATH data using comprehensive SWATH library built from IDA data acquired on different instruments or from publicly available MS/MS data. For our tested human protein spiked with 2% and 10% yeast samples, when a comprehensive generic library was used to extract SWATH-MS data and a cut-off threshold of protein fold change larger than 1.5 with protein area T-Test P-value smaller than 0.05, 3785 human proteins were quantified correctly as not changing in quantities between samples and 155 yeast proteins were correctly quantified as being changed in quantities with false positive discovery rate of 1.37%. We observed that false positive discovery rate would be significantly reduced with more stringent cut-off criteria that measure peptide area T-Test P-values.

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Comprehensive proteomics analysis of vulvar cancer tumour progression

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Vulvar cancer is a rare gynaecological cancer with increasing incidence rate in the past decades, especially among the Indigenous population and human papillomavirus (HPV) prevalent areas. However, due to low incidence rate, only limited numbers of tumour samples are available, here we use complementary mass-spectrometric approaches to identify tumour specific diagnostic and prognostic biomarkers. Mass spectrometry based proteomics analysis for protein discovery has been successfully applied on gynaecological cancers. MALDI-Imaging MS and Label-free quantification MS are able to quantify numerous proteins from different morphological features, which are ideal tools for studying current vulvar cancer proteomics patterns. To investigate vulvar cancer tissue unique proteomics patterns, MALDI-Imaging and LFQ MS have been performed using FFPE tissue blocks from four vulvar cancer patients. Statistics software was used for spectra training and peak matching on different platforms. We have identified 1400 proteins from all samples and half of them are unique to specific tissue types. Based on these analyses, we can discriminate between healthy and tumour and have identified a few proteins which are up regulated during cancer development. Furthermore, RNA-sequencing will be introduced to complement current protein databases with transcriptomic data. In summary, our approach provides valuable insight into vulvar cancer development.

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Tyrosine phosphorylation profiling and phosphoproteome mapping of three mouse tissues

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Reversible tyrosine phosphorylation is a key biochemical process that regulates many responses and is often deregulated in cancer and immunological disorders. In this study, we report a large-scale enrichment and immunoprecipitation of phosphotyrosine-containing peptides from three mouse primary tissues, brain, lung and liver with two commercial available anti-phosphotyrosine antibodies, pY100 and 4G10, to determine complementary enrichment patterns of these antibodies.

In order to obtain more complete phosphoproteome information of each tissue, phosphorylated serine, threonine (pST) and pohsphorylated tyrosine (pY) were enriched via titanium dioxide (TiO_2). We analysed a small portion of these fractions and identified 2256 pST phosphoproteins and 5168 unique phosphopeptides across the three tissues. After pY enrichment, we identified 715, 529 and 274 phosphotyrosine containing peptides and 491, 338 and 203 phosphoproteins from brain, lung and liver tissue samples, using pY 100 and 4G10 antibodies with 1% FDR. Brain tissue had the largest number of unique phosphotyrosine peptides (43.8%) and phosphoproteins (42.7%) of the three tissues examined. Without the pY-enrichment, the distribution of phosphorylated serine, threonine and tyrosine was calculated to be 86.1%, 11.4% and 2.3% for brain tissue, 89.8%, 9% and 1.0% for lung and 84.7%, 12.1% and 2.5% for liver tissue. Combining the identifications from pST and enriched pY-containing peptides, a total of 1683, 861 and 1379 phosphoproteins were identified from brain, lung and liver tissue, respectively. The overlap of pY proteins and pST proteins is around 9% for brain and lung samples and 4% for liver tissue. Each antibody enriched different sets of pY-containing peptides and a recognition specificity analysis shows the 4G10 seems to prefer the sequence window containing negatively charged amino acids. An Ingenuity Pathway Analysis of the pY-containing proteins shows that leukocyte extravasation signaling and integrin signaling are enriched the most in the lung tissue while the Fcγ receptor-mediated phagocytosis in macrophages and monocytes signaling enriched in similar level across the three tissues.

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Analysis of cow's milk intact proteins using liquid chromatography-mass spectrometry.

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Cow's milk is a complex fluid whose proteome displays a diverse set of proteins of high abundance such as caseins (CN) and medium to low abundance whey proteins such as ß-lactoglobulin (ß-LG), a-lactabumin (a-LA), lactoferrin, immunoglobulins, glycoproteins, peptide hormones and enzymes. CNs represent 80% of cow's milk proteins and possess important nutritional and functional properties, whilst ß-LG (largely unknown function) and a-LA (involved in lactose biosynthesis) represents

approximately 10 and 4%, respectively. In addition, a variety of post-translational modifications of these proteins introduces further complexity to the composition of the milk proteome. As genetic variants of CNs and whey proteins influence many properties of milk that are essential to the dairy industry, the development of robust analytical methods for the separation and quantification of variants of milk proteins within single protein fractions is of interest.

Several LC and/or gel (1D- and 2D-PAGE) coupled ESI- and MALDI-based strategies have been applied for the separation and subsequent characterisation of milk protein variants [1-4]. Whilst both top down and bottom up methods could be exploited, the majority of reported analytical workflows have applied bottom-up strategies, due to the high resolution, mass accuracy and the need for large ion fragmentation capability of complex mixtures [1].

This work aims at establishing a robust LC-MS-based method to study intact major proteins from cow's skim milk. The effects of column temperature, mobile phase composition, and gradient conditions were evaluated to improve protein separation prior to analysis using ESI-Qq-TOF MS. High resolution spectra subsequently allowed accurate mass determination and sequencing of major proteins. This top down LC-MS workflow enables a high-throughput profiling of abundant proteins of cow's milk with minimal sample preparation.

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Direct quatitative analysis of native human peptides in complex secretome samples with PEAKS

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Introduction

Native (poly)peptides released into body fluids contain highly relevant information as regulatory biomolecules with both diagnostic and therapeutic potential. A major analytical challenge in secretory peptide researches is the low abundance of the analyte proportional to the large volume of extracellular matrix proteins. The current analytical solution to this problem is to enrich the peptide fraction of a complex sample by physical removal of the most abundant proteins. Here we report a direct approach to quantitative analysis of endogenous peptides in complex secretomes, without removal of highly abundant background proteins.

Methods

A high-resolution mass spectrometric workflow combining database search with de novo sequencing was proposed to facilitate analysis of the secretome beyond the dominant tryptic fragments. After a first round of database searching, with the tryptic enzyme specificity, all confident matches were filtered out. The remaining unidentified MS/MS spectra with good de novo sequencing tags were performed second round database search without enzyme specificity. This allows the identification of endogenous peptides with high sensitivity and accuracy. Peptides were quantified with intensity-based label-free quantification.

Results

Samples were conditioned FCS-containing medium of a selected population of human T-cells, which were reduced, alkylated, fractionated over C4 RP-HPLC, digested with trypsin and run by LC-MS with an LTQ Orbitrap Velos. Given a complex two-dimensional LC-MS/MS dataset (>130 GB) of conditioned medium of two differently treated primary human cell cultures sampled at three time points, PEAKS was supplemented with the described novel data analysis workflow. This allows the identification of small endogenous peptides in the presence of a very busy background of predominant tryptic peptides, which made the former invisible in standard analyses. Label-free quantitation shows that these (poly)peptide profiles contain biomarkers for specific physiological or pathological conditions.

Conclusions

Direct analysis of endogenous peptides in complex secretomes