The proteome in context

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Advances in discovery proteomics have made it possible to map the protein contents of cells to saturation, at least at the resolution of expressed loci. Massively parallel targeting techniques such as Swath/DIA mass spectrometry now support the identification and quantification of thousands of proteins from a sample at a high degree of reproducibility. To gain new biological or clinical insights, to date, the thus quantified proteins have been largely treated as independent entities. However, proteins generally function in context with other biomolecules in the form of macromolecular assemblies. To understand how the proteome as a whole controls, coordinates and catalyzes most biochemical functions of a cell, it is therefore essential to also determine its spatial and temporal organization. We refer to the acute state of a proteome in terms of protein composition and the organization of proteins into modules or complexes as the proteotype. The determination of proteotypes, particularly the organization of proteins into modules and complexes, is therefore an important challenge facing the field of proteomics.

In this presentation, we will discuss emerging computational and mass spectrometric techniques to determine the organization of the proteome and to extract functional information from the results. The ultimate goal of this work is to reach a comprehensive understanding of how the proteotype, considered as a complex system, reacts to genetic or external perturbations and thus determines cellular and organismic phenotypes.

Loss of general O-linked glycosylation in burkholderia cenocepacia results in virulence defects driven by changes in transcriptional control

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Burkholderia cenocepacia is a leading cause of necrotizing pneumonia in cystic fibrosis patients worldwide. In addition to numerous characterized virulence factors, *B. cenocepacia* possesses a general O-linked glycosylation required for pathogenesis in model systems, yet how O-linked glycosylation influences virulence is unknown. Within this opportunistic pathogen, O-linked protein glycosylation is mediated by two non-linked genetic components; the O-oligosaccharyltransferase (*PglL*) and the O-glycan cluster (*OGC*), which are both conversed across Burkholderia species. Using proteomic based approaches, we demonstrate that the loss of O-linked protein glycosylation in Δ*PglL,* Δ*OGC* and Δ*PglL*Δ*OGC* strains leads to multiple changes in known virulence factors such as the flagella apparatus and in transcriptional control/DNA-binding factors. Surprisingly, these observed changes are consistent with inhibition of the known quorum sensing systems cepIR. DNA cross-linking proteomic studies and luciferase assays supports the loss of glycosylation, leading to an altered DNA binding landscape. Analysis of known O-linked glycoproteins demonstrates that only a single glycoprotein is effected by the loss of O-linked glycosylation, a putative lipoprotein we have termed glycosylation sensitive lipoprotein (GSL). Degradomics analysis supports that GSL and other glycoproteins are processed within the periplasm, with the loss of glycosylation promoting protein processing around glycosylation sites. Thus, these studies support a model where the loss of O-linked glycosylation leads to changes in cepIR regulation due to alteration in only in a subset of *B. cenocepacia* glycoproteins. Using proteomic and degradomics approaches we identify a single protein, GSL, which may be responsible for driving the attenuation observed in glycosylation null *B. cenocepacia* strains.

Exploring thiol proteomes with maleimide-based probes and quantitative proteomics

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The X-linked degenerative muscular disease Duchenne Muscular Dystrophy (DMD) affects about 1 in 3500 boys worldwide. Although pathological mechanisms remain unclear, reactive oxygen species appear to be pivotal in exacerbating disease progression. In DMD, we hypothesise that oxidation of the thiol functional groups (-SH) of cysteine residues is affecting the function of proteins to cause cellular and tissue pathology. Through preliminary electrophoretic work with fluorescent maleimide thiol probes, several proteins were found to be more thiol oxidised in muscles of the mdx mouse model of DMD compared with normal mice. To identify proteins undergoing thiol oxidation, we synthesised customised maleimide-based isotopic (^{2}H) mass tags. The identified protein list included titin, a structural protein directly involved in muscle contractile machinery. Titin was oxidised at specific thiol groups that had functional relevance to the weakening of muscle contraction in DMD. My objective is to improve sample preparation techniques and methods of mass spectrometry analysis to identify less abundant muscle proteins undergoing thiol oxidation. We have now synthesised ^{13}C maleimide probes to achieve chromatographic coelution for liquid chromatography mass spectrometry and hence enhance the ability to quantify the amount of thiol oxidation. These probes can also be used with Matrix Assisted Laser Desorption/Ionization (MALDI), which provides a high throughput platform to optimise proteomic workflow for sample preparation. Using MALDI, I have established that standard curves generated with the ^{13}C maleimide probes are not affected by common desalting methods (C18 Ziptip and StrataX). These proteomic techniques can be used in combination to maximise the ability to identify and quantify the oxidation of less abundant proteins.

Temporal quantitative proteomics of human CD4^{+} and CD8^{+} T-cell activation using tandem mass spectrometry and SWATH

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Aberrant T-cell activation or termination is a characteristic of numerous inflammatory and auto-immune diseases. Furthermore, dysfunction of anti-tumour T-cells ('exhaustion') has emerged as an important aspect of cancer development. While some of the signalling events and transcription factors involved in T-cell activation are well-established, a systems map of human T-cell activation circuits remains to be generated. In particular, there are limited quantitative proteomics studies on various human T-cell lineages which have distinct functions. To understand proteomic changes associated with different T-cell lineages, we use a relatively new mass spectrometry-based label free quantification method; SWATH (sequential window acquisition of all theoretical fragment masses) to characterize the temporal changes in activated CD4^{+} and CD8^{+} T-cell proteomes. A consensus spectral library was generated from a set of shotgun T-cell proteomics data with fractionation at cellular, protein and peptide levels. SWATH experiments were then performed at 8 time points following in-vitro T-cell receptor activation over 7 days, on both CD4^{+} and CD8^{+} T-cells subsets from 3 volunteers. A total of 2,100 proteins were quantified and ~500 of these proteins were significantly changed over the course of T-cell activation and termination. Major changes to both CD4^{+} and CD8^{+} T-cell proteomic profiles occurred 24 hours after activation, peaking on the 3rd day. Functional annotation of these significantly changed proteins revealed activation phase-specific modulations of energy metabolism pathways up to Day 3. Thereafter, upregulation of cell death and apoptosis pathways, and downregulation of energy metabolism pathways were revealed as possible regulatory networks associated with termination of T-cell activation. This study generated the first temporal quantitative proteome of human CD4^{+} and CD8^{+} T-cell activation, providing a baseline for future studies.

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Functional analysis of a Campylobacter jejuni nutrient transport protein using proteomics and metabolomics

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Campylobacter jejuni is the leading cause of bacterial gastroenteritis in the developed world. Infection occurs predominantly through the consumption of undercooked/poorly prepared commercial chicken products. Importantly, C. jejuni exists mainly as a commensal organism within the intestines of chickens, but is pathogenic in humans. While the exact mechanism of this difference in pathogenicity is unknown, factors such as motility and nutrient uptake are thought to be significant in the host-pathogen nexus. C. jejuni is generally considered assaccharolytic and primarily utilizes amino and organic acids as carbon sources, with only some strains able to utilize fucose. We conducted label-based LC-MS/MS proteome analysis of C. jejuni NCTC11168O to identify proteins associated with growth in deoxycholate, which mimics gut bile salts encountered during human infection. We quantified 1561 proteins (93% of the predicted C. jejuni proteome). The most significantly induced protein (mean 4.6-fold induction) was the product of the cj0025 gene, which has been previously annotated as a 'putative C4-dicarboxylate transport protein'. To determine the function of Cj0025, metabolomic profiles of media inoculated with C. jejuni wild-type or a Δcj0025 deletion strain were compared, with focus on the uptake of amino acids and Krebs cycle intermediates. Our results showed that the both strains exhibited a preference for six amino acids – asparagine, cysteine, proline, serine, aspartate and glutamate, all of which, bar cysteine, were reduced to less than 15% of their initial concentration in rich media after 48 hours of inoculation. Both strains also showed the capacity to uptake most Krebs cycle intermediates. Therefore, the mutant was not defective for the uptake of any known C. jejuni amino acid or Krebs cycle intermediate carbon source under standard microaerobic conditions. This suggests Cj0025 may have a role in micro-nutrient transport and/or be responsible for transport of a nutrient with more than one active transporter.

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A key role for HtrA in *Chlamydia trachomatis* fatty acid and membrane protein composition

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Chlamydia trachomatis is an obligate intracellular pathogen of significant public health importance, being the leading cause of preventable blindness and bacterial sexually-transmitted infections worldwide. Ocular infection (trachoma) is a neglected tropical disease, with Australia being the only developed country to still have a significant trachoma burden, endemic in remote and Indigenous communities. In addition, urogenital infections can cause serious complications including pelvic inflammatory disease and infertility. Conventionally treated with antibiotics, treatment failure is estimated to occur in 6-10% of cases despite no evidence

of genetic antibiotic resistance in clinical patient isolates. The mechanisms of treatment failure, persistence and pathogenesis in chlamydia are still largely unknown, with genetic tools available for studying *Chlamydia* being limited. The dual chaperone-protease HtrA is conserved in most bacterial species and is involved in the pathophysiology of several pathogens; however, the function of *C. trachomatis* HtrA (CtHtrA) is unclear. Our group recently acquired three mutant *C. trachomatis* isolates which were selected for resistance to an inhibitor of CtHtrA. Whole genome sequencing of the mutants revealed that all isolates had acquired single-nucleotide variations in genes regulating fatty acid synthesis and recycling pathways, with one recently discovered to serve a pivotal role in membrane phospholipid biogenesis. It is therefore our hypothesis that CtHtrA may play a role in chlamydial membrane composition and stability. We aimed to investigate and characterise the phenotype of these mutants to further develop an understanding of CtHtrA function and the impact of its inhibition. Label-free quantitative proteomic analysis revealed significant fold changes in transcription and translation factors, stress response pathways, type III secretion effectors and outer membrane proteins, with an increased abundance of the protein groups in the mutant isolate versus wild-type. These proteomic results could suggest that HtrA has a central role in regulating *C. trachomatis* membrane composition and hence the organism's pathophysiology.

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The mRNA-bound proteome of the early diverging eukaryotic protozoan *Giardia lamblia*

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Post-Transcriptional Regulation (PTR) is a key layer of regulation between an organism's genotype and its functional phenotype. PTR is predominately regulated by (1) the formation of various ribonucleoprotein (RNP) complexes, with RNA binding proteins (RBP) at their core, that repress or calibrate the rate of translation of the messenger RNA; and (2) the splicing and silencing of mRNAs through noncoding RNAs, the spliceosome and RNAi machinery. As such, understanding PTR requires a complex and tiered experimental approach to identify the function and context of each control mechanism, and the specific effectors of regulation in any given circumstance. *Giardia duodenalis* is a major global parasite that causes diarrhoeal disease in more than 200 million people each year. Understanding PTR in this parasite provides insight into how it establishes infection, interacts with the host and develops drug resistance. In addition, as an early-branching eukaryote, *Giardia* provides a model to explore and understand the evolution of PTR mechanisms in the Eukaryota, and represents one of the earliest known species to evolve *Pumillio* domain (Puf) RNA-binding proteins, which are one of the primary classes of translational repression RBPs in higher eukaryotes. Our study aims to identify the RBP repertoire of *Giardia*. We have bioinformatically curated the currently undefined 'RBPome' of *Giardia duodenalis* and analysed their differential transcription during development \and in response to external stress conditions. In addition, we have used a recently developed interactome capture method (Castello et al; 2012) to pull-down and characterize (by LC-MS/MS) proteins bound to poly-A mRNAs in the parasite's replicating stage (the trophozoite) in *in vitro* culture, the first characterization of this kind in any parasitic protist.

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

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Background

Chronic environmental exposure to aluminium has been linked to cancers and neurodegeneration. Through a previous genome wide deletion study, using the model organism *Saccharomyces cerevisiae*, we have demonstrated that deletion of the catalytic subunit (*CKA2*) of the renowned tetrameric protein kinase CK2 results in a tolerant phenotype to Al^{3+}. These findings provided the basis for our proteomic investigations into the mechanisms of Al^{3+} toxicity and detoxification.

Methods

Five strains of *S. cerevisiae* including Al^{3+} sensitive BY4743, *cka1*Δ, *ckb1*Δ and *ckb2*Δ as well as Al^{3+} tolerant *cka2*Δ were treated in duplicate with 1.6 mM Al^{3+} in a time-course (0 to 16 hr). Reverse phase chromatography was used for creation of the spectral library which was generated with ProteinPilot™ 5.0 and searched against the yeast Uniprot 2016 database for protein identification. The spectral library was imported into PeakView™ 2.1 and matched against SWATH-MS data for protein quantification. Statistical analysis of the differentially expressed proteins with a fold change cut-off of 1.5 was performed using Perseus 1.5.5.3.

Results

A total of 3196 proteins (1% FDR) were identified in this study and 2283 proteins were successfully quantified via SWATH-MS. In the Al^{3+} sensitive strains, Al^{3+} was found to damage the cell wall and plasma membrane, disrupts processes such as nucleotide synthesis and repair, amino acid metabolism, ribosome biogenesis and the anti-oxidant response. The key findings of the protective role of *cka2*Δ against Al^{3+} include overexpression of proteins in sulfur metabolism, lysine biosynthesis, anti-oxidants and the heat shock response.

Conclusions

These novel findings provide in-depth understanding of Al^{3+} toxicity and its detoxification, and have implications in finding solutions to Al^{3+}-related problems such as neurodegenerative diseases and cancers.

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N-terminomic dissection of linear ubiquitination and NFkB signalling: Rescuing MALT1 paracaspase immunodeficiency by an allosteric inhibitor

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To specifically enrich for mature protein N-termini and neo-N-termini of proteins we developed 6 and 10-plex TMT TAILS (Terminal Amine Isotopic Labeling of Substrates) (Nature Biotech 28, 281-288 (2010); Nature Protocols 6, 1578-1611 (2011). In analyzing the N-terminome of normal human tissues we find that the N-termini of protein chains *in vivo* can commence at many points C terminal to the predicted start site and result from proteolytic processing to generate stable protein chains: Proteolytic processing generates new protein species with characteristic neo-N termini that are frequently accompanied by altered half-lives, function, interactions and location. We used TAILS to mechanistically dissect a severe human immunodeficiency disease. Paracaspase MALT1 proteolytic activity and molecular scaffolding are key for CARMA1–BCL10–MALT1 (CBM) complex formation. The CBM transduces signaling from lymphocyte antigen receptors. A mutation in MALT1 provided a unique opportunity to identify new MALT1 substrates through positional proteomics using TAILS. We compared B cells from the MALT1[mut/mut] patient with healthy MALT1[+/mut] family members and normal individuals using 10-plex Tandem Mass Tag TAILS with MS3 synchronous peak selection quantification, with and without antigen receptor stimulation. From the MALT1 cleaved neo-N terminal peptide (prime side) and the natural N terminus of HOIL1 identified by TAILS, and the nonprime side of the HOIL1 cleavage site identified by preTAILS shotgun proteomics, we identified HOIL1 of the linear ubiquitin chain assembly complex (LUBAC) as a novel MALT1 substrate. Upon B and T cell receptor stimulation HOIL1 cleavage resulted in disassembly of LUBAC and loss of linear ubiquitination in T and B cells and prevented reactivation of NF-kB signaling. We describe an approach to rescue the mutant MALT1 scaffolding and proteolytic activity in the patient cells. This is the unique example of positional proteomics to mechanistically decipher a human disease, possibly the first disease to be phenotyped by proteomics, and a precision medicine approach to increase patient protease function and so stabilize a patient before life saving bone marrow translatation.

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nUPLC-MS^2 profiling of class I immunopeptidomes of HLA-A2-positive breast cancer patients prioritises antigen selection for tumour-specific immunotherapy

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The success of immune checkpoint inhibition in the treatment of cancer patients demonstrates the potency of utilizing T cell reactivity for cancer therapy. However, varying results between individual patients and severe side-effects highlight the need to increase treatment specificity. Here, we have analysed the immunopeptidomic landscape of 6 HLA-A2-positive triple negative breast cancer (TNBC) patients by nano-ultra performance liquid chromatography tandem mass spectrometry (nUPLC-MS^2) in order to identify tumour-specific antigens that could be used as targets for T-cell mediated therapy. nUPLC-MS^2 profiling identified a total of 35,775 peptide sequences from cancer and adjacent normal tissues. 139 shared, tumour-enriched HLA-peptide sequences predicted to bind HLA-A*0201 were identified in the cohort. Three HLA-peptides that were presented in tumours of all patients originated from Syntenin-1, a protein that is known to be highly expressed in breast cancer cell lines, and is associated with breast cancer progression[1,2], making it an attractive target for immunotherapy in A2-positive patients. We further shortlisted the most relevant tumour-specific source proteins that are presented across the cohort by introducing an average tumour-enhanced cohort presentation coverage (aTeCC), and identified Cofilin-1 (CFL-1), Interleukin-32 (IL-32), Proliferating cell nuclear antigen (PCNA), and Syntenin-1 as antigens with the highest aTeCC values, prioritizing these antigens as targets for immunotherapeutic approaches to breast cancer.

1   Yang, Y. *et al.* Elevated expression of syntenin in breast cancer is correlated with lymph node metastasis and poor patient survival. *Breast Cancer Res* **15**, R50, doi:10.1186/bcr3442 (2013).

2   Koo, T. H. *et al.* Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines. *Oncogene* **21**, 4080-4088, doi:10.1038/sj.onc.1205514 (2002).

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Novel insights in neurodegenerative diseases using advanced imaging, biofluid analysis and microproteomics

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Background.

Understanding the molecular dysfunction of neurodegenerative diseases such as Multiple Sclerosis (MS) or Alzheimers Disease (AD) is of great importance in order to develop new medicines or biomarkers. Disruption of the blood-brain barrier (BBB) and demyelination events plays a major role in disease activity in MS, as indicated by brain magnetic resonance imaging (MRI) contrast enhancement during relapses. Damage of the BBB may be initiated by systemic or CNS inflammation and contribute to escalation of pro-inflammatory responses within the CNS. In AD the behavioral symptoms correlate with the accumulation of plaques and tangles by highly insoluble protein aggregates of A-beta and Tau, respectively. The direct consequence is the damage and destruction of synapses that mediate memory and cognition.

Results & Conclusion

One study investigated the BBB breakdown and active inflammation in MS lesions by MRI followed by correlation analysis of new FLAIRs and Gd-enhancing lesions to plasma phenotype of EVs, plasma proteome and inflammation markers thus allowing description of the systemic inflammation. Several CD molecules and plasma markers could be correlated to the individual inflammatory profiles in the longitutinal study of 1.5 yr. Demyelination dynamics were studied in a cuprizone mouse model of MS by in-depth protein and PTM profiling and correlation to disease stages using microproteomics of brain regions. The study discovered multiple modifications linked to the stages of demyelination. Finally, we present the combination of microproteomics based investigation plaque in combination with AD optimized MALDI MS Imaging for detailed characterization of proteomic alterations in AD brain tissue compared to healthy individuals. By optimerization of the MALDI conditions the detection of proteoforms could be enhanced allowing an improved detection. Similar subregional fractionation of AD tissue allowed significantly improved detection of plaque content.

In summery, our studies have enabled novel insight into both AD and MS using proteomics tools.

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Tissue specific secretomes, the hidden treasure for identification of disease related marker proteins

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Modulations of tissue-specific secretome profiles, triggered by obesity and sedentary lifestyle, or influenced by physical activity are supposed to play a crucial role in the development, prevention and therapy of metabolic diseases including type 2 diabetes.

At the German Diabetes Center we have established an analysis platform to investigate secretomes from insulin sensitive tissues, i.e. adipose tissue, skeletal muscle and liver. To enable comprehensive secretome characterization studies, an adequate sample processing procedures together with different complementary proteomic profiling techniques (e.g. Gel-based and gel-free MS approaches, Multiplex Immunoassays) are the crucial prerequisites. In accordance to that, we developed standard operation procedures and a close quality control to generate high quality samples suitable for comparative analysis. Constantly expanding protein maps, available under Diabesityprot.org, will help to achieve a deeper understanding for the complex and dynamic interplay of proteins involved in the communication between different tissues and its alteration in disease pathophysiology.

Ultimately, tissue specific secretomes, provide a treasure trove of novel disease related marker proteins, which potentially can be used for diagnostic or therapy purpose of multifactorial metabolic disorders.

1. Göddeke S, Kotzka J, Lehr S. Investigating the adipose tissue secretome: a protocol to generate high-quality samples appropriate for comprehensive proteomic profiling. Methods Mol Biol. 2015
2. Hartwig S, Raschke S, Knebel B, Scheler M, Irmler M, Passlack W, Muller S, Hanisch FG, Franz T, Li X, Dicken HD, Eckardt K, Beckers J, de Angelis MH, Weigert C, Häring HU, Al-Hasani H, Ouwens DM, Eckel J, Kotzka J, Lehr S. Secretome profiling of primary human skeletal muscle cells. Biochim Biophys Acta. 2014
3. Weigert C, Lehmann R, Hartwig S, Lehr S. The secretome of the working human skeletal muscle--a promising opportunity to combat the metabolic disaster? Proteomics Clin Appl. 2014
4. Lehr S, Hartwig S, Lamers D, Famulla S, Müller S, Hanisch FG, Cuvelier C, Ruige J, Eckardt K, Ouwens DM, Sell H, Eckel J. Identification and validation of novel adipokines released from primary human adipocytes. Mol Cell Proteomics. 2012;11(1):M111.010504
5. Lehr S, Hartwig S, Sell H. Adipokines: a treasure trove for the discovery of biomarkers for metabolic disorders. Proteomics Clin Appl. 2012
6. Knebel B, Goeddeke S, Poschmann G, Markgraf DF, Jacob S, Nitzgen U, Passlack W, Preuss C, Dicken HD, Stühler K, Hartwig S, Lehr S, Kotzka J. Novel Insights into the Adipokinome of Obese and Obese/Diabetic Mouse Models. Int J Mol Sci. 2017
7. Keipert S, Kutschke M, Ost M, Schwarzmayr T, van Schothorst EM, Lamp D, Brachthäuser L, Hamp I, Mazibuko SE, Hartwig S, Lehr S, Graf E, Plettenburg O, Neff F, Tschöp MH, Jastroch M. Long-Term Cold Adaptation Does Not Require FGF21 or UCP1. Cell Metab. 2017

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Extracting the most out of your multi-omics data

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With the advancement of many high-throughput biotechnologies, an interest of many researchers has been to utilize multiple high-throughput data sources to gain further insights into biology and deeper understanding of complex diseases. In this talk, I will discuss briefly the different ways to interrogate multi-omics data and the different types of questions they addressed. Integration enable scientist to address and ask very specific question utilizing significant testing framework. More commonly, integration also enable us to explore and understand the complex relationships among different molecular phenotypes. I will discuss how rapidly emerging tools in networks research allow us to explore dynamic and static mutation-expression networks through coming exome sequencing and mRNA or protein data. Finally, the modeling of heterogeneity in multi-omics data together with extracting different types of features can help to improve the prognosis of disease outcome.

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Bioinformatics aspects of DIA/SWATH with large extended libraries

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Protein quantitation using DIA/SWATH mass spectrometry relies on using high quality peptide MS/MS spectral libraries, however building such libraries to ensure deep proteome coverage can be time consuming and expensive. In order to address this issue various computational approaches for merging archived or external libraries were created and evaluated, including efforts from our group [1]. Such approaches are appealing, since they promise to expand the set of proteins that can be quantitated via DIA/SWATH, and potentially at low costs, considering the in-silico nature of the process. However, when using larger publicly available reference libraries for extension, the risk of introducing computational artefacts by these approaches can increase as well, and particularly so if the datasets themselves are large.

Here we describe the ways in which SWATH quantitative datasets obtained using local libraries and larger extended libraries can differ, in the context of several proteomics datasets including a recently published large plasma proteomics experiment containing samples from neonates, young children and adults [2]. We also describe a few simple principles that can be used to evaluate the process of library extension itself, in order to ensure that the proteins are reliably detected and their quantitation is consistent and reproducible. These steps are summarised in a recently described workflow [3]. Implicit in it is a filtering of the set of proteins quantitated via this project of extension, which can be used as needed depending on individual project goals.

1. [1] Wu JX, Song X, Pascovici D, Zaw T, Care N, Krisp C, Molloy MP. SWATH Mass Spectrometry Performance Using Extended Peptide MS/MS Assay Libraries. Mol Cell Proteomics. 2016 Jul;
2. [2] Bjelosevic S, Pascovici D, Ping H, Karlaftis V, Zaw T, Song X, Molloy MP, Monagle P, Ignjatovic V. Quantitative Age-specific Variability of Plasma Proteins in Healthy Neonates, Children and Adults. Mol Cell Proteomics. 2017 May;16
3. [3] Wu JX, Pascovici D, Ignjatovic V, Song X, Krisp C, Molloy MP. Improving Protein Detection Confidence Using SWATH-Mass Spectrometry with Large Peptide Reference Libraries. Proteomics. 2017 Oct;17

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Thesaurus: quantifying phosphopeptide positional isomers in DIA experiments

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While mass spectrometry has made a far-reaching impact towards understanding cellular signaling, there is still a huge limitation in analyzing phosphosites occurring in close proximity. Indeed, accumulating phosphoproteomic data shows that phosphorylation sites cluster together in multi-phosphorylated proteins, where over half of sites are within four amino acids of each other. These neighboring sites result in phosphopeptide positional isomers that can sometimes be chromatographically resolved, but because they have the same precursor mass, dynamic exclusion settings often cause these peptides to be overlooked in data-dependent acquisition (DDA) experiments. This, coupled with the stochastic nature of DDA, often results in replicate quantitative experiments that exhibit very poor overlap. Here we propose Thesaurus, a new search engine that detects clusters of phosphopeptide positional isomers from Parallel Reaction Monitoring (PRM) and Data-Independent Acquisition (DIA) experiments. Using the insulin signaling pathway as a model, we demonstrate we can computationally extract distinct quantitative signaling effects of different positional isomers, even if those isomers do not separate chromatographically.

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Working with non-model organisms: an integrated omics workflow for effective assembly of species-specific protein landscapes

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Proteomics approaches are increasingly being employed in a diverse range of fields, particularly with respect to identification of key proteins in non-model organisms. The majority of traditional protein biochemistry has been focused on organisms such as *Homo sapiens* or *Mus musculus*. Successful protein inference relies upon alignment with sequenced genomes and proteins in public domain databases. This proves a significant challenge for the study of non-model organisms for several reasons. Firstly, as such alignments are typically done against large, non-specific datasets, the power of these searches is inevitably negatively

impacted upon. Secondly, the FDR for peptides is increased. Finally, large numbers of mass spectra end up being unassigned, causing numerous novel proteins to be overlooked. Although the simplest solution is to sequence the genome of the study organism, such an undertaking can prove to be prohibitive in terms of cost. We present here a multi-omics workflow that allowed for identification of candidate inner ear proteins from *Acanthopagrus butcheri* (Black Bream), an unsequenced Southern Australian fish. Our approach first involved the sequencing and assembly of transcriptome FASTA using Trinity. Proteomic data collected from the RP-fractionated organic phase of ear stones and endolymph from wild, adult Black Bream were then searched against these FASTA in six frames to create a dataset of transcriptome-matching peptides. Secondly, we performed Blastx analyses of the transcriptome against the RefSeq database, resulting in a dataset consisting of the best aligned proteins from a variety of fish species. These two datasets were merged to create a database to best represent the species under investigation. Finally, mass spectra were searched against this new database and the results integrated with *de novo* sequencing resulting in a comprehensive protein landscape and the identification of novel proteins – many of which would have not been identified using a traditional proteomics approach.

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Galaxy-P: an accessible resource for multi-omics analysis

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Galaxy is a hugely popular bioinformatics workbench that provides bench scientists with access to thousands of open-source tools through a user-friendly interface. The Galaxy-P project and community (http://galaxyp.org/) is an extension of Galaxy that focuses on multi-omic integration, including integrative analysis of mass spectrometry (MS)-based proteomics, genomic and transcriptomic data (1).

Proteogenomics lies at the intersection of proteomics and genomics and is an emerging field of biological research in cancer and other areas. Proteogenomics integrates genomic and transcriptomic data with MS-based proteomics data to directly identify expressed, variant protein sequences. Metaproteomics research on the other hand integrates metagenomic / metatranscriptomic data with MS-based proteomics data to perform taxonomic and functional analysis of expressed proteins. Both these analyses are computationally intensive, requiring many software tools to be integrated into sophisticated workflows which challenges its adoption by non-expert, bench scientists. The Galaxy-P project have recently developed Galaxy-based resources for proteogenomic informatics (2, 3) and metaproteomics informatics (4) with the aim of providing more researchers access to, and training in these multi-omics research fields.

This talk will highlight some of the proteomics tools available in Galaxy and it's new multi-omics capability. It will also discuss how R/Bioconductor tools can be easily integrated into Galaxy, enabling sophisticated statistical analyses to be performed, as well as highlighting other features that make Galaxy an incredibly valuable, accessible resource for bench scientists.

1. https://z.umn.edu/galaxypreferences
2. Chambers, M.C. et al. Cancer Res 77(21), e43-e46 (2017).
3. https://z.umn.edu/proteogenomicsgateway
4. https://z.umn.edu/metaproteomicsgateway

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Identification of multiple plant peptide hormones in secreted peptidome using de novo sequencing sheds light on proteolytic processing and post-translational modifications

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A plethora of gene families encoding known or predicted secreted regulatory peptides occur in diverse plants. Characterised peptides include CLE, CEP, RGF, IDA, PSY, PIP and CIF gene family members, which collectively influence several developmental and defence-related responses. These peptides are secreted at low nM concentrations and act as local or long-distance signals. The peptides are encoded within short open-reading-frames and broadly share a common structure: an *N*-terminal secretion signal, a variable region of unknown function, one or more conserved peptide domains ranging from 5-20 amino acids (depending upon the family). The mature peptides are post-translationally modified. We devised an approach to efficiently elucidate the structures and post-translational modifications of diverse peptide hormones in plant secreted fluids to simultaneously identify and characterise several potential long-distance peptide hormones. We combined an enrichment procedure with mass spectrometry and bioinformatics to comprehensively analyse the secreted peptidome of *Medicago truncatula* root cultures and soybean xylem sap-a fluid that moves to the shoot carrying root-to-shoot signalling molecules (1). Multiple spectra corresponding to four peptide hormone families (CEPs, CLEs, XAPs and CIFs) were found to be secreted from *Medicago* root cultures and present in soybean xylem sap, suggesting that these peptides are long-distance signals. Common PTMs identified included proline hydroxylation and glycosylation and tyrosine sulfation. Several CEP and XAP peptides possessed *C-* and sometimes *N*-terminal amino acids that extended beyond the predicted conserved peptide domain borders and their diverse structures suggested roles for endo- and exoproteases in peptide maturation (1). Several peptides were chemically synthesised to probe the effect of post-translational modifications and variations in length on function. Since root cultures can be made on many plants, they may represent an ideal source to simultaneously and efficiently identify diverse peptide hormones *in vivo* that may play long-distance signalling roles.

1. Patel N, Mohd-Radzman NA, Corcilius L, Crossett B, Connolly A, Cordwell SJ, Ivanovici A, Taylor K, Wlilliams J, Binos S, et al. 2017. Diverse peptide hormones affecting root growth identified in the Medicago truncatula secreted peptidome. Mol Cell Proteomics. DOI: 10.1074/mcp.RA117.000168

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Bioarchaeological proteomics - Identification of proteins from skin and muscle tissue from Ancient Egyptian mummies shows evidence of acute inflammation and immune response

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We performed proteomics analysis of a set of five very small skin and muscle tissue samples collected from three distinct ancient Egyptian mummies of the Old Kingdom (ca. 2200-200 BC). This is one of the first examples of using bioarchaeological proteomics data to uncover clues about life and death in ancient Egypt.

In contrast to most shotgun proteomics experiments, the issue in this work is not managing mountains of data. The proteins in the tissue samples are thoroughly degraded, as evidenced by the extensive smearing present when visualising SDS page gels. This is confirmed by the low number of peptides and proteins identified in the samples. There are typically less than 50 proteins reproducibly identified, whereas a similar analysis of modern human skin samples identifies hundreds of proteins.

We found a large number of keratins and collagens, which was in agreement with our microscopy data, and previous studies showing that collagens are very long-lived. Using nanoflow high-performance liquid chromatography–mass spectrometry of extracted peptides, we identified a total of 230 proteins from the five tissue samples, which consisted of 132 unique protein identifications. We identified numerous proteins indicative of activation of the innate immune response in two of the mummies, one of which also contained proteins indicating severe tissue inflammation, possibly indicative of a lung infection that we can speculate may have been related to the cause of death. The data also suggests that this person may have suffered from chronic inflammatory condition before they died. Taken together, our data provide molecular evidence that certain diseases common in current society were also present in ancient cultures. While sample materials remain incredibly scarce, this study provides a good idea of the level of information which can be produced by such analyses, which will hopefully stimulate further research in the field.

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Near complete mapping of brain neurotransmitters with mass spectrometry imaging directly in tissue sections

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Small-molecule neurotransmitters, their precursors and metabolites are involved in the brain chemical network and transmit signals between neurons. At present, researchers rely on indirect histochemical, immunohistochemical, and ligand-based assays to detect these small-molecule neurotransmitter substances or direct analysis of tissue homogenates using HPLC or LC-MS/MS. Current neuroimaging techniques have very limited abilities to directly identify and quantify neurotransmitters from brain sections. By performing MALDI mass spectrometry imaging (MSI) directly on the surface of a tissue section, the technique has quickly been established as a powerful *in situ* visualization tool for measuring abundance and spatial distribution of endogenous and pharmaceutical compounds, lipids, peptides and small proteins. We recently introduced a reactive MALDI matrix, which selectively targets the primary amine group of neurotransmitters, metabolites and neuroactive substances but also function as a matrix for the ionization (1,2). However, the limitation of using such reactive matrix is its limitation to target all downstream dopamine metabolites derived from monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT) enzymes. The majority of small molecule neurotransmitters such as catecholamines, amino acids and trace amines possess phenolic hydroxyl and/or primary or secondary amines, which are proper nucleophilic groups. We therefore developed a new reactive matrix that can selectively target and charge-tag both phenolic and primary amine groups, thus enabling MALDI-MSI of both MAO and COMT downstream metabolites. By this developed reactive matrix, we were able to detect and map the localization of most of the neurotransmitters and metabolites involved in the dopaminergic and serotonergic network in a single brain tissue section (e.g., in Parkinson's disease) and it represents a novel methodology, which assists their identification through the selectivity of the reaction. The sensitivity and specificity of the imaging approach of neurochemicals has a great potential in many diverse applications in fields such as neuroscience, pharmacology, drug discovery, neurochemistry, and medicine.

1. Shariatgorji M, Nilsson A, Goodwin RJA, Källback P, Schintu N, Zhang X, Crossman AR, Bezard E, Svenningsson P, Andren PE (2014) Direct Targeted Quantitative Molecular Imaging of Neurotransmitters in Brain Tissue Sections. Neuron. 84: 697–707.
2. Shariatgorji M, Nilsson A, Källback P, Karlsson O, Zhang X, Svenningsson P, Andren PE (2015) Pyrylium Salts as Reactive Matrices for MALDI-MS Imaging of Biologically Active Primary Amines. J Am Soc Mass Spectrom. 2015 26:934-9.

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

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Introduction. Stroke is a leading cause of death and disability globally and in Australia. Interestingly, physical activity and exercise is thought to reduce stroke risk; enhance rehabilitation following stroke; and in particular induce remodelling of cerebral blood vessels and their basement membranes, although the mechanisms underpinning these effects remain unclear. Perlecan is a major ECM protein of vascular basement membranes, neuromuscular junctions and cartilage, which is known to be proteolytically processed to release a C-terminal bioactive fragment: the LG3 peptide. Importantly, the LG3 peptide is naturally released in the stroke-injured brain and is profoundly neuroprotective / neuroreparative when administered systemically in animals. We have previously found that the LG3 peptide was increased in the urine of physically active mining workers compared to sedentary controls; however, no previous studies have investigated the relationship between physical activity and the changes in LG3 abundance in human serum where it might have therapeutic benefit.

Aims. To measure the serum levels of the LG3 peptide in response to exercise.

Methods. Venous blood was collected at pre- and post-exercise from six males following an intermittent, high-intensity exercise (85% VO2 max) and a continuous, moderate-intensity exercise (60% VO2 max). LG3 quantification was performed by quantitative mass spectrometry to specifically target the LG3 peptide in serum.

Results. LG3 peptide levels significantly increased in response to moderate-intensity exercise.

Discussion. These results suggest that circulating levels of the neuroprotective / neuroreparative LG3 peptide can be significantly increased under normal physiological conditions in healthy individuals following moderate exercise. We are currently undertaking a pilot clinical study to determine if patients with mild stroke can increase circulating levels of LG3 peptide with moderate to low levels of exercise. These studies may help establish a baseline level of physical activity required to elevate LG3 peptide to potentially beneficial levels in the blood.

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Suppressing proliferation, invasion and non-canonical MAPK signaling by antagonizing the cancer cell surface-restricted uPAR·αvβ6 protein interaction

Mark S. S Baker[1], Subash Adhikari[1], Seong Beom Ahn[1], Abidali Mohamedali[1], David Cantor[1]

1. *Macquarie University, NSW, Australia*

Colorectal cancer (CRC) invades and metastasizes to local lymph nodes or distal organs in late stages, leading to reduction in survival. Current late stage treatments (chemotherapy and radiotherapy) are often ineffective and/or highly toxic. Targeted and immunotherapies hold promise in the treatment of metastasis. We previously discovered an interaction network in commonly Ras-mutated CRCs, involving urokinase plasminogen activator receptor (uPAR). One particularly novel observation was the direct interaction with the cancer cell-restricted integrin αvβ6 (i.e., uPAR•αvβ6). This interaction has been found to play crucial roles in biologies associated metastasis in a high percentage of patients who develop late stage CRC. Here, we rationally designed interference peptides (iPEPs) modelled on uPAR surfaces involved in uPAR•αvβ6 as potential lead antagonists. CRC SW480[β6OE] cells expressing uPAR•αvβ6 were treated with biotinylated iPEPs or scrambled peptides, with binding monitored using streptavidin-FITC and cytoskeleton actin changes with phalloidin. This experiment confirmed only iPEP2 and iPEP6 bound and increased actin "spicule" formation. Matrigel invasion and quantitative cellular proteome changes were performed on SW480[β6OE] (i.e., αvβ6 overexpressing) and naive SW480 cells with/without iPEP treatment. Again, iPEP2 and iPEP6 inhibited growth and invasion, altered morphology, switched key non-canonical MAPK signalling events and significantly changed both the total cell and plasma membrane proteome. A total of 1,358 common proteins were stringently identified across triplicate MS runs between SW480[β6OE] cells with/without iPEPs, of which 37 were downregulated and 67 significantly upregulated (p<0.05, fold change > 2). Pathway analysis showed 88 cancer-related morphology and signalling-related pathway proteins up/down regulated. uPAR•αvβ6 expression switched signalling to favour MAPK-dominant over SMAD pathways and this could be reversed by iPEPs2/6. The uPAR•αvβ6 interactome drives CRC cells to metastasis and antagonising the interaction disrupts processes associated with proliferation, invasion and growth. iPEPs conceivably represent a therapeutic strategy to curb metastasis.

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A label-free quantitative proteomics assessment of osmotic stress responses in *Candida albicans*

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The fungal pathogen *Candida albicans* survives in humans most commonly as a commensal organism in the flora of the gastrointestinal and urogenital tracts, occasionally causing opportunistic infections, such as thrush. However, in people who are immunocompromised, *C.albicans* can cause severe and life-threatening infections. Stress adaptation is critical for the pathogenicity of *C.albicans,* and hence has been studied in detail at the genomic level to try to assess the mechanisms for host-

defence implemented as adaptations to the environments encountered. This study investigated the proteomic changes between *C.albicans* grown under normal conditions and those experiencing salt-stress in the growth media.

Three biological replicates of each condition (three high salt, three controls) were proteolysed with trypsin and the resulting peptides analysed using an ion mobility assisted data independent workflow (LC-IM-DIA-MS). Sample loadings of 100 ng protein on column, were separated over a 90 min linear reversed-phase LC gradient, returning in excess of 1000 protein identifications per acquisition. The data were processed using ProteinLynx Global Server software (vs.3.0.2) to identify and quantify the proteins with a label-free approach. Data were searched against a C. albicans specific database using a 1% false discovery rate (FDR). Gene ontology (GO) functional analysis of the data were queried using blastp, followed by enrichment tests and pathway analysis using R/Bioconductor package systemPipeR (vs.1.10). Data interpretation revealed that a number of central metabolic enzymes are involved with glycerol synthesis, which is a key osmolyte for *C.albicans.*

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Glycosylation pathways associated with disease and personalised approaches to therapies

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Changes in protein glycosylation are associated with most diseases including congenital disorders of glycosylation, autoimmunity and cancer. Technology is an available which enables glycosylation changes to be associated with pathways and disease phenotypes. Determining which glycoproteins and pathways are affected in individual patients and predicting their responses to drug therapies is becoming an increasingly important aspect of personalised medicine. This talk will focus on the technologies needed to access big data sets for individuals and the bioInformatics programs which underpin data interpretation.

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Advances in the Glycoanalytical Toolbox

__Erdmann Rapp__[2, 1], __Rene Hennig__[2, 1], __Samantha Cajic__[2], __Thilo Muth__[1], __Alexander Behne__[1], __Markus Pioch__[2], __Alexander Pralow__[2], __Robert Kottler__[2], __Udo Reichl__[2, 3]

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Glycomics is a rapidly emerging field that can be viewed as a complement to other „omics" approaches. Hence, there is a dramatic increase in the demand for analytical tools and specific databases in glycobiology, respectively, glycobiotechnology. In order to enhance and improve the comparatively small existing glycoanalytical toolbox [1], automated, sensitive, reliable, high-throughput and high-resolution analysis methods including automated data evaluation are required.

Our approach, is the implementation of cutting edge technologies, like multiplexed capillary gelelectrophoresis with laser induced fluorescence detection (xCGE-LIF) and LC-MS in combination with the development of dedicated software (glyXtool[CE] and glyXtool[MS]) to further improve their usability and performance. The development of this high-performance glycoanalysis systems (methods, software and database) and their application to different fields with respect to sample preparation, analysis and data analysis is presented [2-6]. Further, their smart applicability is demonstrated for different types of glycosamples, like biopharmaceuticals, vaccines, human stem cells, milk and blood serum [7-11].

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The role of protein glycosylation on the development of skeletal muscle

__Benjamin L Parker__[1], __Christopher Ashwood__[2], __Jodie Abrahams__[2], __Dena Francis__[1], __James G Burchfield__[1], __Morten Thaysen-Andersen__[2], __David E James__[1]

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The majority of human congenital disorders of glycosylation present with clinical features involving disruptions in organ development especially malfunction of the nervous system and muscles. This suggests that protein glycosylation is vital for proper development and function. However, our understanding of the role of glycosylation, particularly in muscle development, is poorly understood. We hypothesise that glycosylation plays an important role in cell-cell communication during muscle differentiation. Here, we performed a proteomic time-course analysis during muscle differentiation in cultured L6 myoblasts/myotubes. Several glycosyltransferases responsible for terminal galactosylation and sialylation were modulated during myogensis including a down-regulation of B4GALT and ST3GAL, and an increase in ST6GAL1. To investigate whether the altered biosynthetic machinery remodels the *N*-glycome, the muscle differentiation was monitored in cultured L6 cells using glycomics. We observed a decrease in di-galactose-terminating and alpha2,3-linked sialic acid containing glycans, and also an increase in alpha2,6-linked sialic acids containing glycans in end-stage myotubes, confirming a remodelling the *N*-glycome during muscle development. Importantly, these glycome changes were also observed during *in vivo* skeletal muscle development of post-natal mice from day 1-21 with the most notable changes being a decrease in alpha2,3- and increase in alpha2,6-sialylation. To investigate the role of sialic acid linkage switching, we knocked down *st6gal1* using siRNA in cultured myoblasts prior to differentiation. Immunofluorescence microscopy was performed during myogenesis revealing a defect in both myoblast cell fusion and differentiation compared to

scramble siRNA-treated cells. Finally, we performed a quantitative glycoproteomic analysis of muscle differentiation by analysing TMT-labelled glycopeptides by LC-MS/MS employing HCD/EThcD/CID. These data were able to pinpoint changes in specific glycans and localise modification sites on several adhesion molecules and integrins. Taken together, our study will be a valuable resource to further our understanding of the role of glycosylation during muscle development.

Thermal Glycoproteome Profiling: the role of site-specific glycosylation in glycoprotein stability

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It has long been appreciated that asparagine (N)-linked glycosylation of proteins is critical for productive glycoprotein folding and high thermodynamic stability. However, it has remained analytically challenging to investigate the precise details of the site-specific roles of N-glycans in determining the stability of glycoproteins. Thermal proteome profiling has emerged as an efficient mass spectrometry-based method for identifying protein-ligand interactions at a proteome scale. Here, we developed a simple sample preparation strategy to enrich yeast glycoproteins from culture supernatant, and show that this subcellular fraction can be used to assay N-glycosylation occupancy and structure. Further, we used this secretome from yeast with defects in N-glycosylation together with a modified thermal proteome profiling approach to efficiently assay the global consequences of site-specific glycosylation defects on glycoprotein thermal stability.

Uncovering *N*-linked protein glycosylation changes during prostate cancer progression

<u>Rebeca Sakuma</u>[1,2], Daniel Quina[1], Christopher Ashwood[2], Hannes Hinneburg[2], Saulo Recuero[3], Miguel Srougi[3], Katia Ramos[3], Nicolle Packer[2], Morten Thaysen-Andersen[2], Giuseppe Palmisano[1]

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Prostate cancer (PCa) is the second most common cancer in men worldwide. Gleason score classification is the most important predictor of PCa outcomes and is influential in determining patient treatment options. However, tumour heterogeneity, biopsy-sampling error, and variations in biopsy interpretation are still key challenges for accurate prognostication, leading to significant overtreatment with associated costs and morbidity. Changes in the glycosylation profile and the corresponding glycosylation enzymes were previously described to be associated with PCa development and progression, but until this point have been only studied in isolated PCa cell cultures and not in cancer tissues. In this study we aimed to accurately map the *N*-linked protein glycosylation in PCa tissues during disease progression. Membrane proteins were extracted from a cohort of fresh PCa tissue samples, which were accurately grouped into five PCa disease stages (n = 10 per group) as well as tissues from benign hyperplasia patients (n=5). Porous graphitised carbon–liquid chromatography (PGC–LC) and negative polarity ion trap tandem mass spectrometry was used to quantitatively map native *N*-glycans after PNGase F-based release and reduction. A total of 85 unique *N*-glycan compositions and 188 biosynthetically-related structural isomers were detected across the prostate tissues. Complex fucosylated and sialylated *N*-glycans were the most abundant structures. Preliminary data points towards an increase of highly branched (tri- and tetra-antennary) fucosylated and sialylated glycans and a concomitant decrease in high mannose structures during PCa progression. Paucimannosidic glycans were shown with increased abundance across stages of PCa but surprisingly with decreased abundance in end-stage PCa. By integrating the results from the glycome data and lectin blotting analysis with the *N*-glycan biosynthetic pathway, we provide an in-depth and system-wide characterisation of changes in the *N*-glycosylation during PCa progression. These changes can be further explored as potential diagnostic and prognostic marker for PCa detection and development.

Plant glycoconjugate glycans are important in growth and development!

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Glycosylation of plant proteins displays many of the similarities of eukaryotic *N*-glycosylation but also plant-specific *O*-glycosylation through hydroxyproline (Hyp). A class of these Hyp-rich glycoproteins (HRGPs) are the AGPs that are found ubiquitously in plant cell walls, secretions and at the plasma membrane. Most members of this family of proteins are defined by a Hyp-rich protein backbone decorated by type II arabinogalactan (AG) glycans. AGPs are proposed to have a wide variety of functions and play a pivotal role in plant growth and development, particularly in sexual reproduction (reviewed in Ellis et al., *Plant Phys.,* 2010; 153:403-419; Tan et al*., Front. Plant Sci*., 2012; 3:1-10). Pollen exine is essential for protection from the environment of the male gametes of seed-producing plants. We have identified a glycosyltransferase (GT) 31 family member, KAONASHI4

(KNS4)*,* required for normal exine development in *Arabidopsis.* Loss of *KNS4* function results in abnormal exine structure and pollen morphology, reduced fertility and lower seed set confirming that KNS4 is critical for pollen viability and development (Suzuki et al., *Plant Phys.*, 2016; 173:183-205). We have shown that the *kns4* mutant exine phenotype is related to an abnormality of the primexine matrix laid on the surface of developing microspores. Levels of AGPs in the *kns4* microspore primexine are considerably diminished, and their location differs from that of wild type, as does the distribution of pectin labelling. Using a heterologous expression system (*Nicotiana benthamiana*) KNS4 was demonstrated to be a β-(1,3)-galactosyltransferase (GalT) which we believe is responsible for the synthesis of the AG glycans on AGPs.

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Cell wall carbohydrate structure and biosynthesis in pathogenic oomycetes

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The oomycete phylum comprises devastating crop pathogens that represent a serious threat to food production and sustainability. Their cell walls share structural features with both plants and fungi. Like plant cells, oomycete hyphae contain cellulose as the main load-bearing component, whereas chitin, a typical major cell wall component of fungi, occurs in minute amounts in the walls of some oomycete species only. Similar to fungal cell walls, oomycete produce a diversity of β-glucans that consist essentially of β-(1,3) and β-(1,6) glucosidic linkages. Thus, oomycetes represent interesting comparative model systems for cellulose biosynthesis in plants and β-glucan and chitin biosynthesis in fungi. In addition, the enzymes responsible for cell wall biosynthesis in oomycetes represent potential targets of inhibitors that can be used to control the diseases provoked by pathogenic species. However, the proteins associated to the oomycete carbohydrate synthase complexes and their corresponding mechanisms are not well characterised. In this lecture I will present our latest results on the characterisation of the cell wall glycome of ten different pathogenic oomycetes, highlighting species-specific structural epitopes. I will also describe the structural and biochemical properties of the vital membrane-bound glycosyltransferases that form cellulose and chitin in two devastating pathogens, *Phytophthora capsici*, which infects a large number of crops, and the fish parasite *Saprolegnia parasitica*. Different recombinant forms of the enzymes were expressed in heterologous systems and characterised *in vitro* providing insight into the molecular mechanisms and structural organisation of chitin and cellulose synthases.

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Discovery and structure-based characterisation of new-generation influenza virus neuraminidase inhibitors

Benjamin Bailly[1], **Ádám Hadházi**[1, 2], **Linghui Li**[1], **Gael Martin**[1], **Andrea Maggioni**[1], **Thomas Ve**[1], **Jeffrey C Dyason**[1], **Robin J Thomson**[1], **Mauro Pascolutti**[1], **Mark von Itzstein**[1]

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Despite the availability of several drugs to treat influenza virus infection, the pathogen remains a serious threat to human health worldwide. With increasing knowledge of the structure and mechanistic function of the viral neuraminidase, we found potential to develop next-generation neuraminidase inhibitors. We and others have recently reported the discovery of a novel sialosyl sulfonate (**1**), based on the saturated 2-deoxy-*N*-acetylneuraminic acid template, as an inhibitor of influenza virus neuraminidase[1,2]. This compound, which incorporates a sulfonate group in place of the natural carboxylate group, shows much greater inhibitory potency against influenza virus neuraminidase and *in vitro* virus infection than the carboxylate congener. It is also more potent than the benchmark unsaturated inhibitor Neu5Ac2en **2**.
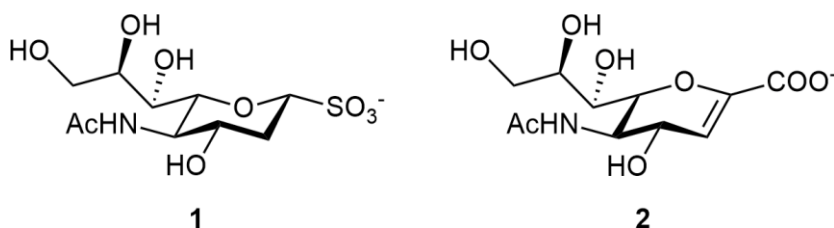
To further explore this new template, we have introduced modifications in place of the ring hydroxyl group that are designed to tailor the compound to the influenza virus neuraminidase active site. We obtained a compound with nanomolar potency against the neuraminidase activity of a panel of human influenza viruses, as well as against *in vitro* infection. Potency remained unchanged against a neuraminidase variant resistant to the influenza virus drug oseltamivir carboxylate, while the compound is not inhibitory to the human Neu2 neuraminidase. X-ray diffraction data of the N2 neuraminidase in complex with the compound revealed a binding mechanism similar to the one of the influenza virus drug zanamivir bound to N2, with slightly distinct features. Together, these results expand the chemical space for the design and synthesis of novel, potent influenza virus neuraminidase inhibitors that may lead to improved antiviral therapies.

1. Hadházi, Á. et al. A sialosyl sulfonate as a potent inhibitor of influenza virus replication. Org. Biomol. Chem. (2017). doi:10.1039/C7OB00947J
2. Vavricka, C. J. et al. Synthesis of Sulfo-Sialic Acid Analogues: Potent Neuraminidase Inhibitors in Regards to Anomeric Functionality. Sci. Rep. 7, (2017).

Cell-line specific glycosylation of respiratory syncytial virus fusion protein and implications for vaccine design

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Unlike pathogens such as bacteria and fungi, the types of glycans on enveloped viruses stem from host biosynthetic pathways and are specific to the cells used during viral replication. Glycosylated surface proteins are incorporated into progeny virions and host cell membranes, and the attached glycans can play multifaceted roles in protein biosynthesis, function and host-virus interactions. These include shielding antigenic epitopes, or acting themselves as epitopes for recognition and neutralisation by the host immune system. The leading vaccine based immunogens for enveloped viruses mimic surface glycoproteins and exhibit native-like antigenic properties[1]. Efforts to develop the first effective vaccine against respiratory syncytial virus (RSV), an important cause of acute lower respiratory infection in infants and immunocompromised individuals, have focused the fusion (F) surface glycoprotein. However, our glycoproteomic studies of F-based vaccine candidates revealed substantially different glycosylation patterns depending on the cell line used. We compared F produced in adenocarcinomic human alveolar basal epithelial cells and human embryonic kidney cells, and highlighted unique glycan motifs on F after production in the latter cell line. Fragmentation of predominate *N*-linked glycopeptides produced glycan oxonium ions consistent with two terminal *N*-acetyl-hexosamine units. These monosaccharide units could potentially represent β1–4-linked *N*-acetyl-galactosamine and *N*-acetyl-glucosamine, commonly called LacdiNAc motifs. These motifs have been identified on a relatively small number of mammalian proteins and have been shown to provoke humoral immune responses and exhibit immunosuppressive activities. Previous structural predictions of F identified an *N*-linked sequon within an antigenic site targeted by potent neutralising antibodies[2, 3]. Thus, F-based vaccines with *N*-glycans containing LacdiNAc motifs may not generate protective antibodies after administration, or may prime aberrant immune responses if the epitopes are not present during natural infection. This work highlights the importance of cell-line choice when producing therapeutics to protect against RSV infection.

1. Horiya, S., MacPherson, I.S. & Krauss, I.J. Recent strategies targeting HIV glycans in vaccine design. Nat. Chem. Biol. 10, 990-999 (2014).
2. McLellan, J.S. et al. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science 340, 1113-1117 (2013).
3. Zhao, M. et al. Discovery of a prefusion RSV F-specific monoclonal antibody that provides greater in vivo protection than the murine precursor of Palivizumab. J. Virol. (2017).

Campylobacter jejuni infection modifies the human intestinal epithelial cell *N*-glycome

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Campylobacter jejuni is a Gram negative microaerophilic bacterium that is the leading cause of food-borne gastroenteritis in developed countries. The common route of human infection is *via* consumption of poorly prepared or under-cooked poultry, in which *C. jejuni* is an asymptomatic commensal. Human disease is characterised by mild to severe inflammatory diarrhoea, vomiting and inflammation. *C. jejuni* has also been associated with post-infection immune-mediated complications such as Guillain-Barre Syndrome, reactive arthritis and irritable bowel syndrome. The molecular basis for *C. jejuni* infection includes initial adherence to, followed by invasion of, human intestinal epithelium; however there remains limited knowledge on the initiation of host-pathogen interaction and subsequent pathogenesis. Here, we investigate the molecular mechanism of *C. jejuni* adherence to human intestinal epithelial cells by identifying changes to the membrane *N*-glycome from the infected host. Human intestinal Caco-2 cells were co-cultured with the pathogenic chicken colonizing isolate *C. jejuni* NCTC 11168O during a time-course of infection. A temporal profile of the Caco-2 membrane *N*-glycome upon *C. jejuni* infection was examined by porous graphitized carbon (PGC) liquid chromatography and tandem mass spectrometry. In total, 175 structural and 59 compositional *N*-glycan features were identified in 48-hour control and infected Caco-2 cells. *C. jejuni* infected cells showed reduction in complex (p<0.007) type glycans and an increase in hybrid (p<0.001) and high mannose (p<0.009) type glycans compared to non-infected time matched control cells. Structural features such as core and outer-arm fucosylation, sialylation and bisecting GlcNAc were significantly reduced (p<0.005) and a number of exclusive glycan structures were found over- or under-represented in Caco-2 cells upon *C. jejuni* infection. *C. jejuni*-mediated modifications of host membrane protein *N*-glycosylation may play important roles in host cell-pathogen interaction and provide further knowledge on the initiation of cellular recognition that leads to colonization of the human intestine.

Modifications Matter: Probing the Effects of Glycosylation on Peptide and Protein Activity

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Glycosylation is the most common co- and post-translational modification of polypeptides, with over 50% of human proteins predicted to display covalently bound glycans. Glycoproteins are known to mediate an array of biological recognition events and

a number of recently approved biopharmaceuticals contain carbohydrate chains (or carbohydrate mimics) that are critical for activity and/or stability.[1] In addition, aberrant glycosylation is associated with a number of disease states including autoimmune diseases and cancer.

The non-templated enzymatic glycosylation process leads to heterogeneous mixtures of isoforms when glycoproteins are produced in eukaryotic expression systems that hinders the ability to study glycoprotein structure and function in a meaningful way. This has led to significant demand for new tools and technologies to facilitate access to homogeneous glycopeptides and glycoproteins to interrogate the role of individual carbohydrate modifications on structure and function. This talk will outline the use of synthetic technologies developed in our laboratory to access homogeneously glycosylated peptides and proteins for structure-function studies and to assess the potential of these molecules as drug candidates.[2] The synthesis and evaluation of glycopeptide cancer vaccine candidates,[3] glycopeptide hormones[4] and thrombin-inhibiting glycoproteins from medicinal leeches[5] will be highlighted.

1.   G. Walsh, R. Jefferis, Nat. Biotechnol. 2006, 24, 1241.
2.   L. R. Malins, R. J. Payne, Curr. Opin. Chem. Biol. 2014, 22, 70
3.   B. L. Wilkinson, S. Day, L. R. Malins, V. Apostolopoulos, R. J. Payne, Angew. Chem. Int. Ed. 2011, 50, 1635-1639
4.   L. Corcilius, A. H. Hastwell, M. Zhang, J. Williams J. P. Mackay, P. M. Gresshoff, B. J. Ferguson, R. J. Payne, Cell Chem Biol. 2017, 24, 1347-1355
5.   Y. S. Hsieh, B. L. Wilkinson, L. C. Wijeyewickrema, R. N. Pike, R. J. Payne, Angew. Chem. Int. Ed. 2014, 53, 3947-3951.

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Twisting tails and curious channels – the phosphoribosyltransferases

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Phosphoribosyltranferases (PRTs) catalyse the transfer of the sugar, ribose 5-phosphate to a nitrogenous base. We have examined catalysis by two PRT enzymes, both of which play key roles in amino acid biosynthesis, and have been identified as new targets for antimicrobial therapeutics.

ATP phosphoribosyltransferase (ATP-PRT) catalyses the first step of histidine biosynthesis resulting in the transfer of a phosphoribosyl unit to ATP. We have carried out kinetic isotope effect measurements to determine the mechanism of this reaction for ATP-PRT enzymes the pathogens *Campylobacter jejuni* and *Mycobacterium tuberculosis*. We are currently using this information to design transition state analogues as inhibitors of this enzyme.

Anthranilate phosphoribosyltransferase (An-PRT) catalyses the formation of phosphoribosyl anthranilate in the biosynthetic pathway for tryptophan. Our results with substrate analogues and inhibitors of An-PRT from *Mycobacterium tuberculosis* reveal how a substrate binding channel both protects an enzyme-bound reactive intermediate and acts as an Achilles heel by providing a mechanism for inhibition.

1 - Mittelstädt, G.; Moggré, G.-J.; Panjikar, S.; Nazmi, A. R.; Parker, E. J. *Protein Sci.* **2016**, *25*, 1492.

2 - Cookson, T. V. M.; Evans, G. L.; Castell, A.; Baker, E. N.; Lott, J. S.; Parker, E. J. *Biochemistry* **2015**, *54*, 6082.

3 - Moggré GJ, Poulin MB, Tyler PC, Schramm VL & Parker EJ. **2017**, Transition state analysis of adenosine triphosphate phosphoribosyltransferase. *ACS Chemical Biology 12*, 2662-70.

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Analytical Strategies for Glycan Characterisation of Biotherapeutics

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Glycosylation is one of the most common posttranslational modifications in proteins of eukaryotic cells. It have been demonstrated that the glycosylation of a protein has a significant effect on the safety and efficacy of recombinant biotherapeutic proteins, with reports of glycosylation affecting the tertiary structure, solubility, stability, immunogenicity, *in vivo* clearance, and bioactivity of the protein. One recent trend is the increasing emphasis on understanding the nature of the different glycan structures involved in the protein glycosylation. Given that various cell lines, expression hosts and protocols can result in different glycosylation patterns, measuring and understanding glycosylation by various analytical techniques is crucial. Furthermore, to understand batch-to-batch variability and to compare recombinant therapeutic proteins with the plasma derived variant, it is necessary to determine where glycosylation occurs as well as the structure and content of the individual sugars. The first task is relatively straightforward using peptide mapping experiments however the latter represents a much more challenging task, and requires a whole suite of MS-based methods coupled with various separation methods.

Overall, in order to support the development and production of new recombinant proteins with improved efficacy and pharmacokinetics and a reduced risk of adverse immunological reactions, there is a regulatory requirement for the comprehensive characterisation of protein glycosylation.

The lecture will give an overview of different methods and technologies used for N-and O glycan characterization. Our major focus is to combine quantitative profiling methods using different label strategies and fluorescent detection in line with mass spectrometry based identification of the separated glycan structures.

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MALDI mass spectrometry imaging of early- and late-stage serous ovarian cancer tissue reveals stage-specific *N*-glycans

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Epithelial ovarian cancer is one of the most fatal gynaecological malignancies in adult women with an estimated 1,580 new cases diagnosed and 1,047 deaths estimated in Australia during 2017. *N*-glycomic studies have extensively reported aberrant patterns in the ovarian cancer tumour microenvironment. Therefore, obtaining spatial information is essential to uncover tumour-specific *N*-glycan alterations in ovarian cancer development and progression. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) was employed to investigate the spatial distribution of *N*-glycans on formalin-fixed paraffin-embedded (FFPE) ovarian cancer tissue sections from both early-stage (n = 3) and late-stage (n = 3) patients. Tumour-specific *N*-glycans were identified and structurally characterised by PGC-LC-ESI-MS/MS, and then assigned to high-resolution images obtained from MALDI-MSI. A total of 14 *N*-glycans were visualised as ion intensity maps while 42 *N*-glycans (including structural and compositional isomers) were identified and structurally characterised. The spatial distribution of high mannose, complex neutral, bisecting and sialylated *N*-glycan families were only observed to be localised to the tumour regions of late-stage ovarian cancer patients relative to early-stage patients. Potential *N*-glycans diagnostic markers that emerged include the high mannose structure, $(Hex)_6 + (Man)_3(GlcNAc)_2$, the bisecting structure, $(Hex)_1 (HexNAc)_3 (Deoxyhexose)_1 + (Man)_3(GlcNAc)_2$, and the sialylated structure, $(Hex)_2 (HexNAc)_2 (NeuAc)_1 + (Man)_3(GlcNAc)_2$. These observations require validation on large patient cohorts, by utilising tissue microarrays (TMAs) as well as evaluation of specific glyco-gene expression levels.

1. Matthew T Briggs, Yin Ying Ho, Gurjeet Kaur, Martin K Oehler, Arun V Everest-Dass, Nicolle H Packer, Peter Hoffmann: N-glycan matrix-assisted laser desorption/ionization mass spectrometry imaging protocol for formalin-fixed paraffin-embedded tissues. Rapid Communications in Mass Spectrometry 03/2017;, DOI:10.1002/rcm.7845
2. Arun V. Everest-Dass, Matthew T. Briggs, Gurjeet Kaur, Martin K. Oehler, Peter Hoffmann, Nicolle H Packer: N -Glycan MALDI Imaging Mass Spectrometry on Formalin-Fixed Paraffin-Embedded Tissue Enables the Delineation of Ovarian Cancer Tissues. Molecular & Cellular Proteomics 07/2016; 15(9):mcp.M116.059816., DOI:10.1074/mcp.M116.059816
3. Ove J R Gustafsson, Matthew T Briggs, Mark R Condina, Lyron J Winderbaum, Matthias Pelzing, Shaun R McColl, Arun V Everest-Dass, Nicolle H Packer, Peter Hoffmann: MALDI imaging mass spectrometry of N-linked glycans on formalin-fixed paraffin-embedded murine kidney. Analytical and Bioanalytical Chemistry 12/2014; 407(8)., DOI:10.1007/s00216-014-8293-7

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Synthetic glycopeptides: versatile tools for glycoproteomics

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Detailed knowledge on glycan composition and their site-specific distribution within a specific glycoprotein is crucial for understanding their complex biological functions. Reliable glycopeptide identification providing concomitant details on both, the glycan and peptide moieties still remains challenging, also because glycopeptides are highly heterogeneous molecules. Defined, synthetic glycopeptides offer a unique opportunity to investigate and validate glycoproteomics sample preparation and analytical workflows and develop novel methods.

We have developed a novel, simplified approach to purify and produce a panel of glycosylated Fmoc-protected Asparagine amino acids carrying *N*-linked glycans with various structures. These building blocks were subsequently used in standard solid phase glycopeptide synthesis to generate a synthetic glycopeptide library containing >100 glycopeptides and their unglycosylated counterparts.

First, a novel, simple, fast and cost-effective technique for HILIC (hydrophilic interaction chromatography) based glycopeptide enrichment ("Drop-HILIC") was developed. Drop-HILIC was used to systematically evaluate the mobile phase effect on ZIC-HILIC (zwitterionic type HILIC) glycopeptide enrichment. We found that glycopeptide enrichment efficiency primarily relied on the applied mobile phase, but we also found that even minimal glycopeptide structure/composition differences already affected ZIC-HILIC enrichment [1].

Glycopeptide ionisation efficiency was investigated using CaptiveSpray Nanobooster™. This allows overcoming possible glycopeptide enrichment biases [1] and reduced glycopeptide ionisation efficiacy [2]. The use of the CaptiveSpray Nanobooster™ itself already resulted in ~5-fold increase in glycopeptide signal intensities compared to conventional CaptiveSpray nano ESI ionisation without any changes to the sample preparation workflow or MS hardware.

Finally, a panel of synthetic glycopeptides was used to evaluate how glycan size and glycosylation site location within a peptide influence ETD fragmentation efficiency and successful MASCOT assignment. The number and quality of assignable peptide backbone fragments was significantly depending on glycan size, the position of the modification within a peptide sequence and the individual precursor *m/z*.

1. Alagesan, K., S.K. Khilji, and D. Kolarich, It is all about the solvent: on the importance of the mobile phase for ZIC-HILIC glycopeptide enrichment. Anal Bioanal Chem, 2017. 409(2): p. 529-538.
2. Stavenhagen, K., et al., Quantitative mapping of glycoprotein micro-heterogeneity and macro-heterogeneity: an evaluation of mass spectrometry signal strengths using synthetic peptides and glycopeptides. J Mass Spectrom, 2013. 48(6): 627-39.

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GlycoStore: a resource for the exploration and annotation of liquid chromatography and capillary electrophoresis glycan data

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GlycoStore (http://www.glycostore.org) is an open access chromatographic and electrophoretic retention database of N-, O- and GSL glycans characterised from a range of glycoproteins, glycolipids and biotherapeutics. It is a continuation of GlycoBase project, but addresses many of the technological limitations, in particular, improvements to the bioinformatics architecture, enhancing data annotations and connectivity with external resources.

The database has four levels. The first level brings together annotated glycomics data sourced from a number of analytical platforms including ultra-high performance liquid chromatography, porous graphitized carbon chromatography with MS detection, and capillary electrophoresis with laser induced fluorescence detection. The second level provides access to a growing, curated database of published literature, with a focus on data that has become available over the past five years, filling an information gap between GlycoBase and GlycoStore. This content includes a number of features such as a detailed comparison of serum glycosylation profiles derived from healthy individuals with different diseases, providing insights into how glycosylation changes are associated with health and disease. The third level is a new search tool that allows users to efficiently filter structure entries based on annotated features (e.g. epitopes and mass), by category type, and glycoprotein. The last level is the provision of dedicated Semantic resources that provides a platform for developers to query and mine the available content as part of the GlyGen project.

Metadata is critical for connecting and reusing glycomics data. Our aim is to create a metadata specification and a glycomic collections registry to facilitate data discovery. We are developing a web-based interface and visualisation tools enabling users to explore GlycoStore, compare data sets, and filter by structural and analytical facets. It also provides the functionality to access supporting data, as well as to visualise regions of interest and connections with data available in other resources.

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Tuning OGT and OGA expression reveals a role for dynamic O-GlcNAcylation in regulating AMPK signaling and autophagy

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In response to injury, cells and tissues remodel their cellular environment to repair damaged structures and if necessary to initiate apoptosis. This process, known as the cellular stress response, includes robust and dynamic changes in the modification of intracellular proteins by monosaccharides of O-linked b-N-acetylglucosamine (O-GlcNAc). Acute enhancment of O-GlcNAcylation reduces apoptosis and necrosis in both *in vivo* and *in vitro* models of injury that include cardiac ischemia reperfusion injury (I/R injury). Although enhanced O-GlcNAcylation suppresses the hallmarks of I/R injury, the molecular mechanism(s) by which O-GlcNAc mediates cardioprotection are largely uncharacterized. The present study sought to determine whether a causal relationship exists between injury-induced O-GlcNAcylation and autophagy, as both cellular processes are induced by ischemic preconditioning and promote cardioprotection. We engineered forms of the O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA), the enzymes that catalyze the addition and removal of O-GlcNAc, that enable the regulation of protein O-GlcNAcylation with biologically inert small molecules. We demonstrate that enhancing wildtype OGT expression is associated with inceased autophagic flux and reduces cell death of H9C2 cells challenged with oxidative stress. Consistent with these data, pharmocological augmentation of O-GlcNAcylation raises autophagosome levels in murine hearts and H9C2 cells. Autophagosome accumulation corresponds to increased proautophagic signaling as indicated by AMP-activated protein kinase (AMPK) and ULK1 activation. The increase in autophagy associated with elevated O-GlcNAcylation was curtailed by AMPK inhibition or deletion, indicating that O-GlcNAc signaling regulates autophagy in part through AMPK. As such, we assessed the O-GlcNAcylation state of these autophagy regulators. AMPKa and ULK1 are O-GlcNAc-modified or associate with O-GlcNAc-modified proteins in a stress-dependent manner. Together, our data suggests that O-GlcNAc can positively regulate autophagy at multiple points along the pathway.

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Design of compounds to block galectin carbohydrate-recognition: In pursuit of eliminating cancer progression

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A number of galectin protein family members have significant roles in promoting cancer and inflammation, some also indicate a role in regulating bone-remodeling, leading to bone loss. Galectins exhibit interactions with cell-surface glycans that can facilitate

disease progression, for example by promoting metastasis in cancer. Designing small molecule compounds that target galectins and compete with their interactions toward endogenous carbohydrates is important in developing potential therapeutics, but is challenging due to characteristics of the carbohydrate-binding site of galectins. This presentation focuses on our structure-based design of small molecules that bind within the galectin carbohydrate-binding site, and thus have potential to reduce their lectin function within biological systems. Our approach to the design of compounds that target galectins includes X-ray crystallographic structure determination of galectins in complex with such blocking agents, giving insight into structural features that are important in the design of potent and selective inhibitors of galectins.

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Glycolipid biosynthesis modulates protein glycosylation in ovarian cancer cells

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Glycan moieties associated with proteins and lipids have been implicated in the regulation of numerous biological and physiological events. We recently observed that altering the (neo-) lacto series biosynthesis in glycosphingolipids (GSLs) impairs α2-6 sialylation on *N*-glycoproteins in ovarian cancer cells[1]. To understand this unexpected interplay between two distinct classes of glycoconjugates, we investigated the role of different glucosylceramide-related GSL series by the deletion of specific glycosyltransferase-encoding genes in ovarian cancer using CRISPR-*Cas9*.

We identified α1-4-galactosyltransferase (A4GALT), β-1,3-*N*-acetylglucosaminyltransferase 5 (B3GNT5) and β-1,4-*N*-acetyl-galactosaminyltransferase 1 (B4GALNT1) as key transferases directly involved in the extension of the lactosylceramide to globo, (neo-) lacto and ganglio-series, respectively.

We successfully deleted these transferases through CRISPR-*Cas9* -mediated gene disruption in the ovarian cancer cell line IGROV1. In addition, the UDP-Glucose Ceramide Glucosyltransferase that catalyses the first glycosylation step in the biosynthesis of GSLs was also deleted to identify its impact on protein glycosylation. The membrane glycans from proteins and lipids were analysed using negative mode porous graphitic carbon liquid chromatography mass spectrometry[2,3].

We observed massive changes in protein glycosylation in all mutant cells, specifically *N*-linked glycans showed several terminal glycan epitopes such as sialic acids, LacDiNac and bisecting GlcNAc that were significantly different. For example, there was an increase in bisecting structures in *ΔA4GALT* and *ΔB3GNT5* cells, while in *ΔB4GALNT1* cells these structures were decreased. The impairment of α2-6 sialylation in *ΔA4GALT* and *ΔB3GNT5* cells was consistent with previous observations, whereas the overall sialylation was increased in *ΔB4GALNT1* cells albeit with diminished α2-6 sialylation.

This is the first ever study of systematically modifying GSL biosynthesis to characterise their effect on protein and lipid glycosylation. GSLs play an important role in moderating cell surface glycosylation and therefore its physiological state, an important factor in ovarian cancer metastasises.

1. Alam, S., M. Anugraham, Y. L. Huang, R. S. Kohler, T. Hettich, K. Winkelbach, Y. Grether, M. N. Lopez, N. Khasbiullina, N. V. Bovin, G. Schlotterbeck and F. Jacob (2017). "Altered (neo-) lacto series glycolipid biosynthesis impairs alpha2-6 sialylation on N-glycoproteins in ovarian cancer cells." Sci Rep 7: 45367
2. Anugraham, M., F. Jacob, S. Nixdorf, A. V. Everest-Dass, V. Heinzelmann-Schwarz and N. H. Packer (2014). "Specific glycosylation of membrane proteins in epithelial ovarian cancer cell lines: glycan structures reflect gene expression and DNA methylation status." Mol Cell Proteomics 13(9): 2213-2232.
3. Anugraham, M., A. V. Everest-Dass, F. Jacob and N. H. Packer (2015). "A platform for the structural characterization of glycans enzymatically released from glycosphingolipids extracted from tissue and cells." Rapid Commun Mass Spectrom 29(7): 545-561.

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Glycosphingolipids trigger reversible transition of mesenchymal and epithelial ovarian cancer cells

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The most described biological program involved in cancer cell dissemination and growth at metastatic sites is Epithelial-to-Mesenchymal (EMT) and its reverse process Mesenchymal-to-Epithelial transition (MET), respectively. This usually confers to epithelial cancer cells being orchestrated by a series of well-described EMT proteins leading to mesenchymal cell features. However, the possible involvement of glycosphingolipids (GSL), cellular components of the cell surface membrane consisting of a ceramide and glycan compartment, has not been investigated in detail.

Here we accessed large ovarian cancer transcriptomic data sets and show that the expression profile of specific genes encoding glycosyltransferases (*e.g. A4GALT* for globosides or *ST3GAL5* for gangliosides) involved in GSL synthesis differs during EMT/MET, coinciding with changes of classical EMT marker (*e.g. CDH1* or *VIM*). Next, we homozygously deleted the globoside glycosyltransferase *A4GALT* (elevated in ovarian cancer cells with epithelial features) using the CRISPR-*Cas9* system. The depletion of globosides was confirmed by LC-ESI-MS/MS and flow cytometry. We further observed an acquisition of

mesenchymal traits as evidenced by *in vitro* as well as *in vivo* assays (anoikis-resistance, enhanced cell motility and dissemination in zebrafish) and enhanced doxorubicin resistance in *A4GALT* knockout cells. Most intriguingly, we provide evidence that E-cadherin-mediated cell-cell adhesion is strictly dependent on enzymatically active A4GALT (experimental setup comprising wildtype, knockout, rescue wildtype, and rescue mutant of A4GALT) and hence on the abundance of globosides. This indicates that specific GSLs exert a pivotal role upstream of E-cadherin. *Vice versa*, mesenchymal cancer cells acquire epithelial traits upon genomic deletion of ganglioside-encoding genes, *e.g.* gain of E-cadherin.

Taken together, our data shed new light into a yet underestimated class of biomolecules during EMT/MET in an ovarian cancer model and propose a pivotal role of GSL during the reversible transition of cancer cells form epithelial to mesenchymal types.

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Protein glycosylation features of metastatic melanoma: the search for prognostic markers

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Metastasis accounts for the majority of mortality associated with melanoma, as limited treatment options exist for advanced disease stages. The identification of prognostic markers for stage III melanoma is of great importance as currently the survival rate for patients at this stage of disease is highly variable. One avenue that has so far been underexplored in melanoma research is the glycosylation pathway.

Here, we investigate the relationship between cell surface glycosylation, metastatic phenotypes, and patient prognosis, as well as report the first in-depth glycan characterisation of cell surface proteins and the Melanoma Cell Adhesion Molecule (MCAM) from metastatic melanoma samples.

The glycosylation profile of tissue from different patient subgroups including good/poor prognosis, primary histology and disease stage were investigated with an aim to identify any subtype-associated glycan features. In addition, the glycosylation profile of cultured cells was compared to patient tissue.

N- and *O*-glycans were released from cell membrane protein fractions of lymph node tumours and cultured cells and characterised using a porous graphitized carbon liquid chromatography mass spectrometry glycomics platform. Structures were fully assigned using MS/MS fragmentation patterns, retention behaviours.

The linkages of the glycan monosaccharides were confirmed using an array of exoglycosidase enzymes and glycan structures were quantitated before and after selected exoglycosidase combinations to confirm differences in structural features including the degree of branching, sialylation and fucosylation. The use of this strategy provided additional valuable structural information including confirmation of the presence of polylactosamine chains, linkages of sialic acid residues and previously unreported epitopes including LacdiNAc carried by cell surface proteins. These structural glycan epitopes may play a role in melanoma metastasis.

This study contributes to our understanding of glycosylation alterations in melanoma metastasis, towards using specific glycosylation changes as novel biomarkers for monitoring metastasis and predicting prognosis.

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Whoa man! Unexpected protein O-mannosylation pathways

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Until recently protein O-Mannosylation in yeast and metazoans was thought to be directed by a single family of conserved orthologous protein O-Mannosyltransferases (pmts/POMTs). The family of pmts is essential in yeast, and in humans the orthologous POMTs underlie a subgroup of congenital muscular dystrophies designated α-dystroglycanopathies that are caused by loss of O-Mannosylation of a-dystroglycan. Recently, novel types of O-mannosylation in yeast and in metazoans were discovered using gene engineering and lectin-enriched O-Mannose glycoproteomics, and it appears there may be more to come. Analysis of the yeast O-Mannose glycoproteome pointed to a novel nucleocytoplasmic type of O-mannosylation resembling the nucleocytoplasmic O-GlcNAcylation found in eukaryotic cells except yeast[1, 2], although the yeast enzyme(s) responsible for the O-mannosylation is still unknown. In higher eukaryotes O-mannosylation of cadherins was found to be independent of the POMTs[3, 4], and using a CRISPR/Cas9 genetic dissection strategy combined with sensitive and quantitative O-Man glycoproteomics, we identified a novel family of TMTCs encoded O-Mannosyltransferases[5]. Congenital deficiency in the TMTC3 gene was shown to underlie Cobblestone Lissencephaly with brain malformation. The *TMTC1-4* genes were imperative for cadherin and protocadherin O-Man glycosylation, and using combinatorial knockout of *TMTC1-4* distinct roles of the individual isoenzymes for specific b-strands of the cadherin EC domains were identified. In addition, O-Man glycosylation of IPT/TIG domains of Plexins and hepatocyte growth factor receptor (HGFR) was not affected in TMTC KO cells, suggesting the existence of yet another O-Man glycosylation machinery. Our study demonstrates that regulation of O-mannosylation in higher eukaryotes is much more complex than envisioned.

1. Neubert, P. et al. Mapping the O-Mannose Glycoproteome in Saccharomyces cerevisiae. Molec Cell Prot 15, 1323-1337 (2016).
2. Halim, A. et al. Discovery of a nucleocytoplasmic O-mannose glycoproteome in yeast. Proc Natl Acad Sci USA 112, 15648-15653 (2015).
3. Vester-Christensen, M.B. et al. Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. Proc Natl Acad Sci USA 110, 21018-21023 (2013).

4. Larsen, I.S.B. et al. Mammalian O-mannosylation of cadherins and plexins is independent of protein O-mannosyltransferases 1 and 2. J Biol Chem 292, 11586-11598 (2017).
5. Larsen, I.S.B. et al. Discovery of an O-mannosylation pathway selectively serving cadherins and protocadherins. Proc Natl Acad Sci USA 114, 11163-11168 (2017).

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Identifying regulators of O-GlcNAcylation during injury

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In the 30 years since the discovery of nucleocytoplasmic glycosylation, O-GlcNAc has been demonstrated to regulate protein function by modulating characteristics that include protein folding, localization, degradation, activity, post-translational modifications, and interactions. The cell coordinates these molecular events, on thousands of cellular proteins, in concert with environmental and physiological cues to fine-tune epigenetics, transcription, translation, signal transduction, the cell cycle, and metabolism. The cellular stress response is no exception: diverse forms of injury result in dynamic changes to the O-GlcNAc sub-proteome that promote survival. To date, the majority of studies have focused on identifying proteins and pathways regulated by O-GlcNAc that combat cytotoxicity. Currently, little is known about the regulation of the two enzymes that write and erase the O-GlcNAc-modification, the O-GlcNAc transferase (OGT) and the O-GlcNAcase (OGA). To provide insight into the regulation of OGA, the enzyme that catalyzes the removal of O-GlcNAc, proximity biotin ligation (BioID) in combination with Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) was used to define the basal and stress-induced interactome of OGA. This analysis revealed 90 interaction partners of OGA, many of which exhibited increased binding to OGA upon stress. The associations of OGA with fatty acid synthase, OGT, heat shock cognate 70 kDa protein (HSC70), filamin-A, and heat shock protein 27 (HSP27) were confirmed by co-immuno-precipitation. Furthermore, the pool of OGA bound to FAS demonstrated a substantial reduction in specific activity (~85%), suggesting that FAS is a novel protein inhibitor of OGA. Consistent with these observations, overexpression of FAS augmented O-GlcNAcylation of a subset of cellular proteins. Collectively, these data support a model in which protein interactors of OGA, such as FAS, regulate its local activity and thus lead to remodeling of the O-GlcNAc-sub proteome in a manner consistent with cell survival.

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High-throughput phosphoproteomics: technologies and applications in systems biology

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Mass spectrometry (MS)-based proteomics has been used to study global and dynamic cell signalling for over a decade and phosphoproteomics has become a burgeoning field in its own right. Developments over the last few years have steadily increased the scope of phosphoproteomics studies in molecular biology, but major challenges remain. Chief among these are practical limitations in scaling, performance and reproducibility. However, these limitations are now falling away, with the development of robust and reproducible workflows aimed at simplifying phosphoproteomics experiments. The 'EasyPhos' method now enables the rapid and accurate acquisition of large numbers of phosphoproteomes, facilitating the analysis of dynamic and *in vivo* signalling. Very recent developments to the workflow now ensure high performance in sample-limited conditions, while simultaneously reducing sample preparation time. For example, protein precipitation steps have been eliminated, reducing opportunities for sample loss and variability. Together with sensitivity-boosting optimisations this has yielded a 300% improvement in performance of the method under sample limited conditions. Collectively this now enables experiments from 200 μg of cell lysate for a depth of >10,000 quantified phosphopeptides in 1 h of MS measurement time. I have recently applied these technologies to wide ranging biological questions, including the investigation of GPCR Kappa Opioid signalling in both a time- and brain-region resolved manner, and unravelling the temporal complexity of stem cell differentiation. The Kappa Opioid study has provided the first systems-view of kappa opioid receptor (KOR) signalling *in vivo*, revealing new mechanisms of drug action. In mice treated with KOR agonists, we identified a novel mechanism to abolish the major known side effect of aversion while preserving beneficial therapeutic analgesic and anticonvulsant effects. Excitingly, these and several other studies are collectively revealing unprecedented regulation of the phosphoproteome, emphasising the central role of post-translational regulation in proteome plasticity.

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Trans-splicing of class I HLA bound peptides diversifies the immunopeptidome

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Human leukocyte antigen class I (HLA-I) molecules sample the proteome following the degradation of intracellular proteins via the proteasome and other proteolytic mechanisms. These complexes of HLA-I and peptide (pHLA) are then recognized by T cells and the nature of the bound peptide ligand is the key driver of adaptive immunity. The diversity of these displayed peptides, the immunopeptidome, plays an essential role in maximizing T cell responses. Recently it has been reported that a large fraction of

HLA-I peptides are derived from spliced non-contiguous sequences (fusion peptides), which are not templated linearly in the genome. In order to identify such peptides, several assumptions were used that restricted potential HLA-I ligands to being derived from the same antigen and between two sequences in close proximity (proximal *cis*-splicing). Here we develop a sophisticated data-driven workflow to demonstrate that fusion peptides are generated through distal and proximal *cis*-splicing as well as *trans*-splicing (where distinct proteins contribute peptide segments). In addition, we find that ligation occurs predominantly between two segments of similar length and describe a new category of peptides (termed polybrid) of unknown origin. Importantly, these fusion peptides display canonical binding sequence features, although on average they had lower predicted affinity for the restricting HLA-I alleles. These results highlight the complexity and diversity of HLA-I peptide display and broaden our understanding of potential targets of T cell immunity and have important implications for vaccine design and immunotherapy.

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Quantitative N-Terminomics and Phosphoproteomics reveal distinct signalling networks governing regulated necrosis of neurons in excitotoxicity

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Excitotoxicity, initiated by over-stimulation of ionotropic glutamate receptors (iGluRs), is a major pathological process directing regulated necrosis of neurons in both acute and chronic neurological disorders. Upon over-stimulation, iGluRs allow massive influx of calcium ions into the affected neurons, leading to over-activation of two groups of neurotoxic calcium-dependent enzymes: (i) the cysteine proteases calpains, which catalyse limited proteolysis of specific neuronal proteins to modulate their functions and (ii) neuronal nitric oxide synthase (nNOS), which generates excessive NO to induce oxidative damages. The calpain-proteolysed proteins and the NO-induced oxidative damages in turn modulate the activities of proteases, protein kinases and phosphatases to perturb the expression and phosphorylation of specific neuronal proteins. Presumably, these perturbed proteins form signalling networks that direct neuronal necrosis. To define these signalling networks, we aim to identify the calpain substrates and the perturbed proteins in neurons undergoing excitotoxic cell death. Using the Terminal Amine Isotopic Labelling of Substrates (TAILS) proteomics method, we identified the exact sites of cleavage in ~300 neuronal proteins proteolytically processed by calpains and other proteases activated in neurons undergoing necrotic death. Additionally, using the stable isotope dimethyl labelling method, we definitively identified ~1300 neuronal proteins and ~1000 phosphosites in neurons undergoing excitotoxic cell death. Among them, around 150 neuronal proteins exhibited dynamic changes in abundance and/or phosphorylation levels in response to glutamate over-stimulation. Bioinformatic analysis revealed that some of the calpain substrates and neuronal proteins exhibiting significant changes in phosphorylation levels form distinct signalling networks. Using biochemical approaches, we found that some components of the predicted signalling networks induce neuronal death by aberrant regulation of key protein kinases critical to neuronal survival. Taken together, our findings illustrate how results of quantitative proteomic analyses can form the conceptual framework for investigation to define the molecular mechanism governing regulated necrosis of neurons.

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A Genetic Dissection Approach to Functional Glycomics

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Glycosylation is one of the most abundant and diverse posttranslational modifications of proteins, but arguably also one of the most difficult to study with respect to occurrence, structure, and biological functions. Heterogeneity in occupancy and structures of glycans on proteins is the major obstacle for analytic strategies, and this is also the major obstacle for exploiting biological functions of glycans in biotechnology. We have therefore taken genetic deconstruction & reconstruction approaches to dissect the glycosylation and modification processes of proteins and proteoglycans. We use rational combinatorial knockout/in of relevant genes with precise gene editing tools (DNA nuclease "scissors" ZFNs and CRISPR/Cas9) to simplify[1], dissect[2], and design[3] glycosylation more or less at will. The strategy has led to increased knowledge of different glycoproteomes as well as discovery of new types of protein glycosylation[4, 5]. Genetic dissection of glycosylation in isogenic cells enables exploration of biological functions of specific glycosylation features, and the generated libraries of cells may be used to develop cell-based glycan arrays for interrogation of interactions with glycans. We will present illustrative examples of the power of gene editing in the glycosylation field that is rapidly turning into "Lego-Toying".

1. Steentoft, C. et al. Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nature Methods* **8**, 977-982 (2011).
2. Schjoldager, K.T. et al. Deconstruction of O-glycosylation-GalNAc-T isoforms direct distinct subsets of the O-glycoproteome. *EMBO Rep* **16**, 1713-1722 (2015).
3. Yang, Z. et al. Engineered CHO cells for production of diverse, homogeneous glycoproteins. *Nature Biotech* **33**, 842-844 (2015).
4. Halim, A. et al. Discovery of a nucleocytoplasmic O-mannose glycoproteome in yeast. *Proc Natl Acad Sci USA* **112**, 15648-15653 (2015).
5. Larsen, I.S.B. et al. Discovery of an O-mannosylation pathway selectively serving cadherins and protocadherins. *Proc Natl Acad Sci USA* **114**, 11163-11168 (2017).

From genome to glycome: Automated analytical workflows to align glycomics with other data to gain insight into complex biological systems

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Glycosylation is a key post-translation modification for most secreted and cell surface proteins as well as many biological drugs that are designed to modify the immune system and inflammatory pathways. Monoclonal antibodies are amongst the available drugs to address major medical challenges including cancer and autoimmunity, transplantation and the use of stem cells. Major improvements in separations technologies that focus on intact glycoproteins, glycopeptides and released glycans have been driven by the needs of the pharmaceutical industry to manufacture safe and efficacious biological drugs as well as new opportunities that are opening up in basic research.

Detailed structural analysis and accurate quantitation are vital for the analysis of the glycans attached to biopharmaceuticals at all stages of production from understanding the roles for the glycans through clonal selection and production, downstream processing and QC.

In basic research the relevance of glycosylation to understanding the complexity of the systems biology of disease is now widely recognised. Linking the glycome of serum or individual glycoproteins proteins directly to genetics, epigenetics, transcription, metabolomics and proteomics is now feasible. Precision/personalised medicine, including mapping changing glycosylation of disease related glycoproteins, is now recognised for its importance in clinical decision points, such as determining which patients will benefit for particular treatments.

Identification of the plant Golgi localized UDP-GlcNAc transporter and its role in endomembrane lipid and protein glycosylation

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Glycosylation reactions require activated glycosyl donors in the form of nucleotide sugars to drive processes such as post-translational modifications and polysaccharide biosynthesis. Many of these reactions often occur in the endomembrane using cytosolic-derived nucleotide sugars, which are actively transported into the lumen by nucleotide sugar transporters (NSTs). We recently identified a plant UDP-GlcNAc transporter responsible for the delivery of substrate for the maturation of N-glycans and sphingolipids within the endomembrane. To determine the biochemical phenotype of the UDP-GlcNAc transporter loss-of-function mutants, we have applied both proteomic and metabolomic approaches. Initially we developed a reliable N-glycopeptide enrichment and mass spectrometry-based analytical workflow to detect, identify and quantify N-glycopeptides. Next, we applied lipidomic approaches to profile sphingolipids from loss-of-function mutants. Analysis of omic data indicated that that N-glycopeptides containing complex N-glycans (e.g. GlcNAc) only comprise about 5% of the N-glycopeptide population in mutant lines. In contrast, N-glycans from wild-type plants are comprised of around 35% complex-type N-glycans i.e. those containing GlcNAc. While sphingolipid analysis indicated that GlcNAc containing lipids comprised less than 10% of that observed in wild type plants. Our findings indicate that the reference plant Arabidopsis contains a single UDP-GlcNAc transporter responsible for the maturation of complex N-glycans and sphingolipids in the Golgi lumen. The work also highlights the detailed insight and resolution that can be achieved using modern omic approaches.

Detailed glycomics and glycoenzyme transcriptomics of amyotrophic lateral sclerosis blood-derived monocytes

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Brain tissue-resident macrophage-like cells, microglia, are innate immune cells of the brain. Monocyte-derived macrophages (MDM) from peripheral blood are known to benefit the resolution of local brain inflammation involving hyper-activated microglia in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). Altered protein glycosylation is known to contribute to or arise from many immune-related diseases, but it still remains unknown if aberrant glycosylation features of monocytes and MDMs are associated with ALS. Herein we explore these important immune aspects of ALS.

We obtained detailed *N*- and *O*-glycan profiles of extracted proteins of peripheral pan-CD14+ monocytes and corresponding MDMs from a cohort of ALS patients and healthy individuals using porous graphitised carbon liquid chromatography ion trap tandem mass spectrometry. Glycan isomer characterisation and determination of their relative abundances were determined and statistically compared between ALS and healthy individuals. Gene expression levels of enzymes relevant for *N*- and *O*-glycosylation were obtained using RNA sequencing to assess any regulation of the glycosylation machinery of ALS monocytes.

More than 100 glycans were characterised from the investigated monocytes and MDMs. The *N*-glycan structures were mainly of the diantennary, complex type carrying various glyco-epitopes such as a2,3-/a2,6-linked neuraminic acids and core and Lewis-type fucosylation. Furthermore, high mannosylation was also an abundant monocytic feature. Only minor qualitative and quantitative differences were detected between the glycan profiles of monocytes derived from ALS patients and healthy

individuals, which were supported by relatively constant mRNA expression of the biosynthetic *N-* and *O-*glycosylation enzymes between these two patient groups.

This is the first study to explore the exact glycosylation signatures in the peripheral immune cells central to ALS. Our findings are part of our aim to advance the understanding of the cellular features and disease mechanisms underpinning ALS and associated neurodegenerative diseases.

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SWATH analysis of human plasma glycopeptides without predefined glycan compositional knowledge

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Glycoproteomics investigates glycan moieties in a site specific manner to reveal the functional roles of protein glycosylation. Identification of glycopeptides from data-dependent acquisition (DDA) relies on high quality MS/MS spectra of glycopeptide precursors and often requires manual validation to ensure confident assignments.To explore alternative acquisition strategies, we investigated the utilities of pseudo-MRM using MRM_HR and data independent acquisitions using SWATH for glycopeptide analysis. These approaches allow data acquisition over the full MS/MS scan range allowing data re-analysis post-acquisition, without data re-acquisition. The advantage of MRM-HR over DDA for N-glycopeptide detection was demonstrated from targeted analysis of bovine fetuin where all three N-glycosylation sites were detected, which was not the case with DDA. To overcome the duty cycle limitation of MRM-HR acquisition needed for analysis of complex samples such as plasma we trialed DIA. This allowed development of a targeted DIA method to identify N-glycopeptides without pre-defined knowledge of the glycan composition, thus providing the potential to identify N-glycopeptides with unexpected structures. This workflow was demonstrated by detection of 59 N-glycosylation sites from 41 glycoproteins from a HILIC enriched human plasma tryptic digest. 21 glycoforms of IgG1 glycopeptides were identified including two truncated structures that are rarely reported.

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An Integrated 'Omics' Approach toward Understanding the Role of Aberrant Lipid Metabolism in Colon Cancer

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Lipids play important physiological roles in the regulation of cellular homeostasis, including as structural and functional components of cellular membranes, for energy storage, and as intra- and intercellular signaling molecules. Emerging data indicate profound dysregulation of cellular lipid metabolism and signaling in colorectal cancer, and there is an increasing recognition of their contributions to malignancy and metastatic progression. However, a detailed survey of the global 'lipidomic hallmarks' of colon cancer, and comprehensive structural characterization of the multitude of isomeric lipid species that may be functionally involved in regulating the interconnected networks and causal relationships between gene, transcript, protein and lipid species on cellular phenotype, is currently lacking. Furthermore, the clinical potential of colon cancer associated lipidomes as diagnostic or prognostic biomarkers of the disease, or as targets of therapeutic intervention, remain largely unknown. Here, to address these needs, I will describe results from our recent studies aimed at the development of mass spectrometry based workflows for comprehensive lipidome analysis, including the characterization of isomeric unsaturated lipids differing only in the locations of their C=C double bonds, and quantification of differences in their abundance between patient matched tumor and normal tissue samples and within a series of molecularly-annotated colon cancer cell lines. The power of integrating transcriptomic, proteomic and lipidomic datasets will also be described for interpreting the functional consequences of these differences in global lipidome inventories, and to determine whether variations in lipidome profiles explain part of the clinical heterogeneity of the disease and constitute a distinct, or complementary, taxonomy relative to currently used genomic and transcriptomic classifications.

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Understanding how proteome foldedness changes under proteostasis stress

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Cells have an extensive quality control network responsible for maintaining proteostasis. This network regulates protein synthesis, folding and transport. After decades of dedicated examination, the folding and stability characteristics of many individual proteins are well understood *in vitro*. However, understanding the kinetic process of protein folding in cells remains a grand challenge, whereby much of the proteome folds through discrete steps at different quality control checkpoints. We have recently developed a fluorogenic thiol-binding dye (TPE-MI) that can capture a snapshot of the balance of unfolded protein relative to folded states in intact live cells (Chen et al. 2017, Nat. Comm.). This approach does not require any expression of specific protein reporters, and has the potential to offer single-protein kinetic folding information for endogenous proteins at a proteome-wide scale. Here, we describe the application of this probe to determine proteome foldedness in cells following a variety of

stressors. This knowledge will contribute to our understanding of disorders characterised by proteostasis imbalance, and will assist in targeting those proteins most prone to misfolding under stress conditions.

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Integrated proteomics and lipidomics profiling of plasma cells in multiple myeloma patients: A pilot study

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Multiple myeloma (MM) is the second-most common haematological malignancy and is characterised by the clonal expansion of abnormal plasma cells within the bone marrow, as well as the production of aberrant monoclonal immunoglobulin. Though durable remission is possible, MM has traditionally been considered incurable, with relapse occurring in almost all patients. Currently, prognosis is determined using a score derived from serum markers (lactate dehydrogenase, beta-2-microglobulin and albumin), as well as cytogenetic abnormalities in the plasma cell clone. With 7 new treatment agents being approved by the FDA since 2012, novel prognostic tools are needed to guide therapy for individual patients. In this pilot study, we developed a multi-omics approach to investigate molecular differences among different risk groups as well as between relapse and newly diagnosed patients. Plasma cells were isolated using CD138 microbeads from patient bone marrow aspirate samples and then subjected to proteomics and lipidomics profiling. Interestingly, while both proteomics and lipidomics results were able to separate relapse from non-relapse patients, the molecular profiles of plasma cells were virtually indistinguishable among traditional prognostic risk groups. A down-regulation trend was observed in relapse compared to non-relapse patients in both proteomics and lipidomics profiles. Out of ~4300 identified proteins 172 were down-regulated while 22 were up-regulated. Untargeted lipidomics showed similar pattern with 187 features down-regulated and 94 up-regulated. Lipid set enrichment on targeted lipidomics indicated significant down-regulation of phosphocholines. Proteomics pathway enrichment revealed significant down-regulation of sphingolipid de novo biosynthesis in relapsed plasma cells, and up-regulation of TCR and NF-kB signalling. This pilot study supports the feasibility and utility of a full multi-omics study in plasma cells to further understand the systems changes in relapsed MM, with a goal of developing biomarkers for detecting and predicting relapse, guiding the therapeutic use of currently available treatments, as well as new therapy development.

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Metabolomics-based investigation of the mechanism of action of novel bis-triazine antimalarials reveals perturbation of arginine methylation

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Malaria is a major global health burden, responsible for over 200 million cases annually, and new antimalarial medicines are urgently needed to treat this potentially-fatal parasitic infection. The malaria parasite, *Plasmodium falciparum*, has developed resistance to all currently-approved antimalarials, which underscores the critical need to discover new drugs with novel mechanisms of action. The bis-triazines represent a novel class of antimalarial compounds that were identified in a high-throughput phenotypic screen and further optimised to exhibit potent antimalarial activity. Importantly, these compounds are based on a novel chemical scaffold, but the mechanism of action of is unknown, which limits further development of this series. The aim of this study was to reveal the mechanism of action of bis-triazine antimalarials.

An untargeted metabolomics approach using high resolution accurate mass LC-MS was implemented to reveal the impact of bis-triazine treatment on the cellular biochemistry of *P. falciparum*. Incubation of *P. falciparum*-infected red blood cells with a potent bis-triazine induced a unique metabolic profile that differed from other known antimalarials. A dose-dependent accumulation of dimethyl-arginine was the most significant unique metabolic perturbation observed in treated cells. Levels of related metabolites, including monomethylated arginine and lysine, were also increased following compound exposure. Stable-isotope tracing and proteomic studies confirmed the perturbation of methylation pathways, and fluorescent labelling of bis-triazine analogues using click chemistry revealed localisation in the parasite nucleus. Overall, this work reveals a novel mechanism of action for the bis-triazine antimalarials, leading to aberrant protein methylation within the parasite.

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Application of quantitative proteomics to' leaky-gut' and intestinal barrier dysfunction - shifting landscapes in the understanding of IBD pathophysiology

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The aetiology and cure for inflammatory Bowel Disease (IBD) are uncertain. Measures reflectingto disease activity, permeability, and Inflammatory state are available but are limited in their ability to predict or measure 'clinical remission' - a newly accepted gold-standard for treatment of this condition. Scientists and clinicians are embracing the concept of intestinal epithelial barrier integrity and its role in the pathogenesis and natural history of IBD.

Peptide biomarkers from the low-mass-plasma proteomes have been identified as significant players in the diagnosis of IBD, the differentiation of active disease and remission, and remission and healthy individuals. These markers have been quantitated using label-free and absolute MRM techniques. Binding partner studies show a novel relationship to endocytic signaling, lipid metabolism and actin nucleation; and additionally correlate to the 'tissue integrity' of leaky-gut' IBD patients.

Modulated proteins in patients with ongoing intestinal damage may be able to predict for relapse and the need to escalate treatment. Markers which can be translated into treatment management able to measure repair of leak, restitution and epithelial cell healing are being sought to manage IBD.

1. Wasinger VC, Yau Y, Duo X, Zeng M, Campbell B, Shin S, Luber R, Redmond D, Leong RW. 2016. Mol. Cell. Prot. 15,1: 256-265.
2. Yau, Y.Y., Duo, X., Leong, R,W., Wasinger, V.C. 2015. Mol. Cell. Prot. 14,2:441-454.
3. Chang, J., Leong, R.W., Wasinger, V., Ip, M., Yang, M., Giang Phan, T. 2017. Gastroenterology. S0016-5085,17:35731.

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Exosomes and implantation - new insights into endometrial-embryo interaction

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Communication between a developing embryo and hormonally-primed endometrium is essential to achieve implantation and establish pregnancy. Importantly, the point-of-first-contact between the embryo and the maternal-endometrium occurs at the endometrial luminal epithelium. We highlight for the first time a unique insight into the developmental biology of embryo implantation – investigating cellular and secreted changes important for receptivity and implantation, and the contribution of exosomes in regulating this microenvironment. Utilising a combination of cell models, targeted physiologically relevant treatments, and quantitative proteomics, we demonstrate endometrial epithelial cellular and secreted protein changes in response to ovarian steroid hormones that drive development of the endometrium to become 'receptive' to an embryo, and to the blastocyst-derived hormone, human chorionic gonadotrophin, which enhances endometrial changes essential for receptivity and implantation. We demonstrate that exosomes (40-150nm nanovesicles) released from endometrial epithelial cells are an important component of these interactions during receptivity and implantation. Utilizing proteomic profiling we defined the protein constituents of purified endometrial epithelial-derived exosomes influenced by menstrual cycle hormones estrogen and progesterone, revealing significant reprogramming associated with cell adhesion, migration, invasion, and extracellular matrix remodeling. In addition to hormonally-treated endometrial cell/secreted and exosomal proteins changes, all findings were validated in human primary uterine epithelial cell-derived material (cells/secretome/exosomes). Functionally, exosomes were internalized by human trophoblast cells and enhanced their adhesive capacity; a response mediated partially through active focal adhesion kinase signaling. Together, our results illustrate the dynamic intracellular and secreted protein changes in the endometrium and responses to the pre-implantation embryo, and an active contribution of exosomes to regulating this environment, that together provide key insights into mechanisms of change associated with human implantation and establishment of pregnancy.

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Multiplex targeted proteomics assay for single-shot flavivirus diagnosis

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Publish consent withheld

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Proteomic profiling of predictive biomarkers for hypoxia-activated prodrugs in head & neck squamous cell carcinomas

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Hypoxia is a ubiquitous feature of many tumours contributing to disease progression and treatment resistance, and therefore represents a well-validated physiological target for cancer therapy. Several hypoxia-activated prodrugs have been developed at the University of Auckland. However, further clinical development requires identifying tumours that are hypoxic, express prodrug-activating reductases, and are intrinsically sensitive to the activated drug. Profiling of gene expression typically uses RNA-based

methods, but most phenotypes are more directly linked to the protein expression. Here we employed a targeted proteomics strategy to develop an assay for candidate prodrug-activating reductases and endogenous markers of hypoxia with a group of housekeeping proteins for nomalisation. By employing stable isotope-labelled peptide standards we performed absolute quantitation of the proteotypic peptides in head and neck squamous cell carcinomas (HNSCC) cell lines and xenografts. The results showed variable reductase expression across a HNSCC panel and corresponding mouse xenografts, with CYB5R3 and NQO1 exhibiting high expression, and POR, AKR1C3, TXNRD1 and FDXR in an intermediate range. T This is consistent with the observation that *CYB5R3, NQO1* and *TXNRD1* transcripts are amongst the most abundant mRNAs encoding prodrug-activating reductases across two HNSCC clinical cohorts. Seven detectable endogenous hypoxia markers, which have been clinically validated at the mRNA level as hypoxia classifiers in HNSCC, showed protein upregulation under chronic hypoxia in cell culture. In conclusion, the multiplexed assay is suitable for assessing absolute levels of reductase expression in cell lines and tumours, and there is promising potential to integrate additional biomarkers for hypoxia and genes that determine intrinsic sensitivity in the future to build powerful predictive biomarkers assay capacity to support drug development and clinical application.

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Development of a high resolution LC-IM-MS platform for comprehensive structural analysis of glycosphingolipid head groups and its application in breast cancer glycobiology

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Glycosphingolipids (GSLs) are amphipathic lipid molecules comprised of a hydrophobic lipid tail and hydrophilic glycan head group. GSLs expressed on cell membranes can alter in response to external stimuli and disease, making them potential markers and/or targets for cellular disease states. Analytical challenges associated with GSL glycan head groups arise from a high degree of compositional similarity. Structural differences in linkage, branching and anomericity must be distinguished from each other, with the added complexity of the presence of isobaric isomers. Analysis using hydrophilic interaction high performance liquid chromatography (HILIC-HPLC) is one of the most common methods for the analysis of released and fluorescently labelled glycans, and provides relative quantitation based on fluorescence detection. The co-elution of structures however can also create ambiguity in structural assignment. The use of ion mobility spectrometry (IMS) as an additional level of separation, based on molecular shape and recorded as collision cross section (CCS), can aid characterisation of isomeric structures. In the work here, we present a workflow for the analysis of procainamide-labelled GSL glycans using HILIC-UPLC-FLR with IMS-MS/MS using a Waters ACQUITY UPLC H-Class and Synapt G2-S. We have created an experimental reference database of GSL glycan standards containing glucose unit (GU), *m/z*, CCS values and MS/MS spectral profiles for each glycan in all observed ion states. The use of these different attributes in matching experimental data for glycan identification was assessed using in-house developed software. This database was employed in the analysis of genotypically different breast cancer cell lines, in order to identify glycan changes and potential therapeutic targets. Overall, this technology offers a sensitive and robust way of characterising and quantifying glycans which can be applied to the discovery of glycan markers in a range of clinical settings.

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Improving the MALDI fragmentation pattern of complex and intact disulphide bonds with aniline

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Characterization of peptides containing intact disulphide bonds (DSB) via mass spectrometry is challenging. DSB are key post-translational modifications in proteins and highly important in the overall stabilization of protein structure. Mapping these DSB and determining the cysteine residue pairing delivers crucial information in protein characterization. Although applications to decipher DSB are not limited to antibodies (biologicals), they may be the major driver in pushing the field forward, as mapping of disulphide patterns in antibodies is crucial to determine the structural characteristics of the protein therapeutics. While most electrospray (ESI)-based applications mostly provide large-scale automated analysis, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is applied to quickly check the cross-link presence. Our study demonstrates that the addition of aniline to alpha-cyano-4-hydroxycinnamic acid as matrix allows a more efficient detection and fragmentation of peptides containing DSB in non-reduced proteins. We report how incorporating aniline in a common MALDI matrix, alpha-cyano-4-hydroxycinnamic acid, improves both DSB detection in MS as well as DSB peptide fragmentation behaviour by MALDI-TOF-TOF compared to other additives and matrices. This improved disulphide assignment will be a significant new tool for when a simple screening to confirm the DSB existence is required.

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Creating a 3D-LC/MS method for proteomics by introducing displacement mode chromatography as an application for spectral library extension

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Displacement chromatography has been used as a preparative technique to enrich proteins [1], but it has not been explored extensively for proteomic applications. The method works by highest affinity binders being retained at the top of the column, while lower affinity peptides are pushed further down. After applying the displacer molecule which has a higher affinity than any peptide in the sample, these zones are pushed down the column forming a displacement train. The eluting peptides show a rectangular chromatographic profile of high purity. Displacement on an SCX column has the advantage over gradient elution that no salt is required but rather a suitable displacer is used, providing complementarity with online LC-MS analysis. In this work we explored the use of online displacement chromatography to augment standard 2D-LC workflows of peptide separation, thereby establishing a 3D-LC method for proteomics.

We used a simple LC setup of two valves to place the SCX column in front of the analytical reverse-phase column. A pool of two prostate cancer cell lines was separated using offline high pH reversed-phase chromatography to generate 13 fractions. Each of these fractions was loaded onto the SCX-Trap column. One displacement step was used to separate the doubly and triply charged peptides, to recover the column an ammonium acetate step was used which recovered the higher charged peptides. This approach was compared to the standard 2D method.

Using our 2D-LC workflow we identified 2335 proteins consisting of 14029 peptides of which 317 were found to be acetylated on N-terminus. In our 3D-LC approach we identified 3137 proteins (+34%) consisting of 23528 peptides (+68%) of which 613 (+93%) were acetylated on N-terminus. These results show that employing a 3D separation we identified significantly more peptides which leads to a more comprehensive spectral library for enhancing label free MS quantitation.

1. R. Freitag, J. Breier, Displacement chromatography in biotechnological downstream processing, Journal of Chromatography A, 691 (1995) 101-112

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High Resolution - Mass Spectrometry Cellular Thermal Shift Assay (HR-MS-CETSA)- post-translational modifications impact on thermal protein stability

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Observation of the proteins thermal stability towards increasing temperature forms the foundation for methods that explore thermally induced protein unfolding. Heat treatment induced protein unfolding and aggregation can be graphically presented as a sigmoidal melting curve which allows estimation of melting temperatures (Tm). It was shown that ligand engagement changes the functional state of the protein and induces thermal shift (Tm). This is the principle of thermal shift assays (TSA) and recent extension of this method - Cellular Thermal Shift Assay (CETSA). CETSA principle takes into account the fact that biophysical thermal stability (thermal induced unfolding) of individual proteins can be monitored and quantified in lysates/intact cells/tissue samples. MS based CETSA tackles thermal shifts in whole proteome using quantitative mass spectrometry. To further extend CETSA application in drug target deconvolution, here we proposed high resolution (HR) MS-CETSA to increase a target specificity.

Accordingly, we investigated further impact of post-translational modifications on the protein stability. Following the data analysis, we were able to detect in the sample with limited input more than 5000 distinct phosphorylation residues in the single experiment. It corresponds to more than 5000 unique melting profiles and or ITDR (isothermal dose responses). We have confirmed our hypothesis that phosphorylated proteins exhibit different stability towards increasing temperature. Overall the data points towards destabilizing effect of the phosphorylation, although detailed mechanism has to be further determined. Furthermore, presented method could help to identify functional phosphorylation sites in the protein which occupancy would be reflected in protein stability.

The Cellular PTM resolved MS-CETSA has potential to add a very valuable dimension to current pre-clinical and clinical drug development defining drug target engagement and off target effects in relevant cell and tissue systems.

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Discovery DIA: All Ion fragmentation on the timsTOF Pro

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Data Independent Acquisition (DIA) strategies, where all peptides are systematically fragmented using mass-isolation windows, are being rapidly adopted for quantitative proteomics. These approaches provide robust quantitative data, but are generally at the expense of discovering new peptides from samples being analysed. While a few DIA approaches also acquire MS1 information, all of these approaches are fundamentally limited by current MS technology, resulting in slow cycle times and reduced duty cycle. In particular, with improvements in chromatography (producing <4 sec FWHM peaks) now providing significant gains in sensitivity and peak capacity, current DIA sampling rates (~2.5 – 3.5 sec) are too slow for reliable and accurate quantitation. Furthermore, the use of narrow isolation windows (4 - 9m/z windows) restricts the duty cycle to a small fraction of the total ion current.

The recently developed trapped ion mobility spectrometry (TIMS) from Bruker adds an innovative dimension to quadrupole time-

of-flight (QTOF). The second generation dual TIMS analyser in the timsTOF Pro has advantages over other IMS-QTOF configurations, where ions are released dependent on their mobility from the second section of the TIMS analyser, while the further incoming ions can be accumulated in parallel in the first part of the TIMS analyser. This parallel accumulation theoretically allows duty cycles of up to 100% to be achieved, with no ion loss. Additionally, the MS1 mobility is encoded in the MS2 data, facilitating mobility correlated extraction of MS1 and MS2 features, generating spectra that can be identified with conventional database search algorithms. Overall, this approach provides a comprehensive unbiased acquisition of samples at or near 100% duty cycle, but without the need for a spectral library.

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The RNA binding proteome in time and space

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In the majority of cells, mRNA species are spatially located and translated in a highly controlled manner involving the interaction of cis- and trans- acting factors with RNA-binding proteins (RBPs) (Harvey, WIRE RNA, 2018). Aberrant translation of proteins in the wrong subcellular location can give rise to multiple disease states such as Spinal Muscular Dystrophy, Fragile X, and autism.

We have developed a suite of methods, based around LOPIT (localization of organelle proteins using isotope tagging), to determine the three dimensional organisation of the proteome in eukaryote cells with high resolution (Mulvey, Nature Protocols (2017)). We have applied these methods to many different biological systems, including mapping protein relocalization upon perturbation. Intriguingly, we consistently observe that up to half the proteome cannot be discretely assigned to a single localisation. This observation has been recently supported by comparing two very different approaches to map proteins to their subcellular niches (Thul, Science (2017)).

To gain insight into the relationship between the steady state location of the translatome and its control though RBP interaction, we have built upon the above methods to create a highly efficient capture method, OOPS (orthogonal organic phase separation) which enriches UV crosslinked RNA and protein. This method samples interface between aqueous and organic solutions to enrich RBPs in a manner which is compatible with downstream proteomics and RNA sequencing. OOPS is able to recover all the protein bound (PBR) and free RNA in an unbiased way, and from the same sample, all the crosslinked RBP and free proteins. We have applied this method to multiple biological systems identifying the majority of previously identified RBPs but also, novel groups of RBPs. Sequencing of the PBR indicates that essentially all long RNAs in the cell are bound by proteins, enabling the identification of protein binding sites of the full transcriptome via read coverage.

We have also adapted the LOPIT method to be compatible with capturing the spatial location of RBP and PBRs to create spatial maps.

These methods will be reviewed and the implications of spatial translation on cellular mechanisms reviewed.

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Global mapping of Salmonella-host protein-protein interactions

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To ensure survival and proliferation inside a host, bacterial pathogens inject a cocktail of toxic effector proteins directly into the host cell cytosol. Once injected, these effectors modify a broad range of host target proteins to facilitate pathogen survival. The intracellular pathogen *Salmonella* Typhimurium (STm) injects ~30 different effector proteins into host cells, however, knowledge of their targets is sparse. To systematically resolve the molecular interface between STm and the mammalian host during active infection, we infected macrophages with a library of STm strains expressing affinity tagged effector proteins from the endogenous chromosomal locus, followed by large scale Affinity-Purification and quantitative Mass-Spectrometry. In addition to validating several previously described protein-protein interactions, we uncovered a plethora of novel effector-host protein-protein interactions and, surprisingly, several novel effector-effector interactions. These findings provide the first molecular details of a complex host-pathogen interaction during active infection and have broad implications for the molecular basis of functional redundancies commonly observed for bacterial effector proteins.

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The MLKL pseudokinase domain controls protein activity and oligomer formation during necroptotic cell death

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Necroptosis is a cell death mechanism characterised by permeabilisation of the inner plasma membrane, with subsequent release of cellular contents initiating an inflammatory response. The pseudokinase, Mixed Lineage Kinase-domain Like (MLKL) is the most terminal known effector of necroptotic cell death. MLKL is a multi-domain protein in which the N-terminal four-helix bundle (4HB) executes cell death via lipid engagement and is tethered to the C-terminal pseudokinase domain (PsKD) by a two-helix linker. Following cell death stimuli, MLKL is phosphorylated by Receptor Interacting Protein Kinase-3 promoting activation and

oligomer formation, which is essential for necroptosis. The stoichiometry, structure and dynamics of MLKL during transition to an active oligomer remains unclear. Using native mass spectrometry, we determined that MLKL assembles as a tetramer where as a mutant MLKL (^{E351K}MLKL) remains monomeric. Assembly of tetramers upon membrane engagement was further characterised using Fast-Scan Atomic Force Microscopy. To determine the structural changes between monomeric ^{E351K}MLKL and tetrameric MLKL, we utilised cross-linking mass spectrometry with constraint mapping and small angle x-ray scattering to model the monomer and tetramer MLKL structures. By combining these results with hydrogen-deuterium exchange mass spectrometry we mapped the dynamic changes that occur as MLKL transitions to an active state. This analysis revealed that the PsKD is the molecular switch that constrains the 4HB, while the linker facilitates oligomerisation upon activation. We next validated our observations by reconstituting *MLKL$^{-/-}$* cell lines with mutant MLKL proteins designed to disrupt interaction sites identified from our analysis. Together, this work describes how the PsKD of MLKL regulates the transition to an active tetramer during necroptotic cell death and identifies potential pharmacological targets in inflammatory pathologies.

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Global analysis of cell signaling networks by quantitative proteomics

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Mass spectrometry (MS)-based quantitative phosphoproteomics is a powerful technology for global analysis of cellular signaling networks. In particular, understanding the dynamics of tyrosine phosphorylation (pTyr) is of great importance in eukaryotic cells due to its crucial role in regulating intracellular signaling networks controlling cell fate decisions such as proliferation, migration, differentiation, cell cycle progression and apoptosis. I will present how we optimized and employed quantitative phosphoproteomics technologies to delineate receptor tyrosine kinase (RTK) signaling dynamics activated by different growth factor ligands leading to differential cellular outcome. Reproducible workflows based on phosphopeptide enrichment using TiO2 or pTyr-specific antibodies in combination with label-free quantitation and LC-MS/MS analysis on Q-Exactive Orbitrap type mass spectrometers allowed us to quantify thousands of phosphorylation sites and derive their kinetic profiles as a function of ligand and stimulation time. Using this strategy, we revealed RTK-specific phospho-regulation of key adaptor and signaling molecules, which fine-tune cell migration and proliferation. Based on a multidisciplinary approach, which combines quantitative phosphoproteomics and functional cell-based assays, we identified ligand-dependent mechanisms for the control of RTK signaling and for the specification of long-term cellular outcomes. Our findings underscore the importance of investigating receptor tyrosine kinase signaling networks in a ligand-dependent manner to identify the key regulatory phosphotyrosine sites, which can determine cell fate decisions.

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Pathogenesis of celiac disease: Identification of isopeptides by LC-MS/MS

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Celiac disease (CD) is one of the most frequent food intolerances affecting approximately 1 % of the population. CD is triggered by the ingestion of gluten from wheat, rye or barley. The intestinal tissue transglutaminase (TG2) plays a key role in the complex pathogenesis of CD, because it forms, inter alia, covalently linked complexes with gluten peptides, which induce the formation of antibodies against these complexes. These antibodies in turn contribute to an increased adaptive immune response. [1]

The aim of this study is to identify the binding sites between TG2 and gluten peptides.

The binding sites of the gluten peptide-TG2-complexes consist of intermolecular N$^\varepsilon$(γ-glutamyl)lysine bonds, so-called isopeptides.[2] For the development of an analytical strategy to identify isopeptides, samples of the digested complexes and of a negative control were measured with a nano-LC-MS/MS system in triplicate and the data were analyzed by the two proteomic software tools MaxQuant and Skyline. Within MaxQuant the data were searched for peptides and isopeptides, comparing samples and negative controls. Subsequently, the MaxQuant output data and the raw data were analyzed with Skyline to visualize the differences. In the next step, the measured MS/MS-spectra were searched for the calculated masses of the product ions, which were then assigned to the peptide by the software.

These experiments on the identification of isopeptides were focused on the development of a suitable strategy, whereby an analysis method with a model system and an isopeptide standard were achieved. Furthermore, four isopeptides between a microbial transglutaminase and the model peptide could be identified, first by manual search in the data sets and secondly by combining the proteomics tools MaxQuant and Skyline.

1. Schuppan D, Junker Y, Barisani D, Gastroenterology 2009; 137: 1912-1933
2. Fleckenstein B, Qiao SW, Larsen MR et al., J. Biol. Chem. 2004; 279: 17607-17616

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Proteomic analysis of 4-phenylbutyrate treated HepG2 cells stably expressing ATP-binding cassette transporter A1 (ABCA1) mutants

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The ATP-binding cassette A1 (ABCA1) transporter is a cellular membrane protein that exports cellular cholesterol to form high density lipoprotein (HDL) and protect against cardiovascular disease (CVD). Many mutations in ABCA1 disrupt trafficking to the plasma membrane and reduce its function. We previously showed that the chemical chaperone, 4-phenylbutyrate (4-PBA) can restore membrane localisation and increase the cholesterol efflux function of mutant ABCA1s.[1] The aim of this study was to identify proteins regulated by 4-PBA that restores ABCA1 localisation. Two ABCA1 mutants (p.T1512M and p.N1800H) expressed in stably transfected HepG2 cells were subject to 4-PBA treatment. The cell lysates were prepared from 4-PBA treated and untreated samples and differentially regulated proteins were analysed by sequential window acquisition of all theoretical fragment ion spectra-MS (SWATH-MS). Proteins showing a >1.5 fold change were subject to bioinformatic analysis using STRING and Panther software packages to categorise their function and potential interactions with ABCA1. This analysis identified a number of trafficking and chaperone proteins including transmembrane protein 33, syntaxin-binding protein 2, transmembrane emp24 domain trafficking protein 2, transmembrane emp24 domain trafficking protein 5 and HSP90 co-chaperone that could assist ABCA1 in restoring its plasma membrane localisation. These proteins warrant further characterisation for their potential to rescue ABCA1 localisation.

1.   Sorrenson, B., Suetani, R. J., Williams, M. J. A., Bickley, V. M., George, P. M., Jones, G. T., & McCormick, S. P. A. (2013). Functional rescue of mutant ABCA1 proteins by sodium 4-phenylbutyrate. Journal of Lipid Research, 54, 55-62.

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Investigating the substrate recognition motifs of Hmt1 and PRMT1

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Arginine methylation is a widespread and functionally-important post-translational modification (PTM) found on over 700 eukaryotic proteins. Methylation of arginine residues has been shown to modulate protein-protein interactions involved in many cellular processes, including transcriptional regulation, RNA processing, signal transduction, and DNA repair. The majority of arginine methylation in the *Saccharomyces cerevisiae* proteome is catalysed by the protein arginine methyltransferase (PRMT), Hmt1, and its mammalian orthologue PRMT1 similarly accounts for most arginine methylation in the human cell. For the past thirty years, the arginine-glycine-glycine (RGG) motif has been widely recognised as a canonical substrate recognition sequence for both Hmt1 and PRMT1. Despite the functional importance and diverse substrates of these enzymes, their complete substrate recognition motifs have, until now, remained undefined. To this end, we employed a novel motif analysis technique to quantitatively and comprehensively investigate the effects of different amino acid residues in nearby positions on Hmt1- and PRMT1-mediated methylation, and thereby elucidate their full substrate recognition sequences. Our novel motifs revealed that, while the 'RGG' sequence is still central to PRMT substrate specificity, this model is oversimplified and does not adequately portray the complex intricacies of this process. We have, for the first time, directly confirmed the importance of aromatic residues in the +3 position for Hmt1 activity. Our results also highlighted Hmt1's intolerance for the negatively-charged, acidic amino acids in the upstream positions, an observation strengthening evidence for methylation-phosphorylation crosstalk. Compared with its yeast counterpart, PRMT1 exhibited slightly broader specificity, likely indicative of its more numerous cellular substrates. The refined motifs determined through this study provide unique insights into the molecular mechanisms of substrate recognition employed by these enzymes, and may therefore inform the development of novel therapeutic agents targeting PRMT1, or facilitate the prediction of additional Hmt1 and PRMT1 substrates.

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

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Inflammation is the body's response to injury or infection. A hallmark of inflammation is the accumulation of leukocytes, which remove pathogens and necrotic tissue by phagocytosis and proteolytic degradation. Leukocytes/monocytes are mainly recruited by chemokine activation of chemokine receptors, resulting in leukocyte morphological changes, extravasation into the inflamed tissue and chemotaxis along the chemokine gradient to the site of injury or infection. while monocyte chemoattractant proteins (MCPs) and chemokine receptor 2 (CCR2) is the major pair, involving this process and contributing to the pathogenesis of atherosclerosis, obesity and type 2 diabetes. CCR2 is known to signal via G protein and β-arrestin-mediated pathways, the downstream signalling pathways have not been thoroughly explored.

Protein phosphorylation and dephosphorylation are crucial for cellular signal transduction. Dynamic regulation of reversible, site-specific protein phosphorylation is critical to the signalling networks. Here, we performed a data-independent acquisition (DIA) based proteome and phosphoproteome quantification workflow to investigate signal transduction and regulation in MCP-1-activated CCR2-expressing cells. This workflow showed excellent reproducibility and quantification accuracy. More importantly, in addition to some canonical signalling pathways, such as MAPK, JAK/STAT and Akt/mTOR, we have mapped and manually curated other signalling networks, including Rho guanine nucleotide exchange factors (ARHGEFs), nuclear pore complex (NPC) proteins and actin cytoskeleton. Most of the characterised and quantified phosphopeptides in these networks have never been linked to MCP/CCR2 signalling. Phosphorylation kinetic study confirmed the dynamics phosphorylation regulation of the canonical networks; furthermore, it provides the phosphorylation kinetics information of ARHGEFs, NPC and actin cytoskeleton, the consequence of activating which matches the biological function of MCP/CCR2 signalling- guiding cell migration. In light of the accurate quantification and high reproducibility provided by DIA, this study provides new insights into MCP/CCR2 signalling and may guide the identification of potential therapeutic targets.

Using MALDI-IMS to explore the distribution of peptides in Australian sea anemones: *Oulactis* spp.

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Secreted peptides from sea anemones are of interest as novel therapeutic leads for the treatment of autoimmune, as well as other, diseases. One such peptide is proving successful in clinical trials as the basis of a drug to treat psoriasis.[1] Despite the growing interest in sea anemone peptides, there is a lack of knowledge regarding those found in endemic Australian species. Moreover, little is known regarding the distribution of peptides in discrete morphological regions of sea anemones, and the correlation, if any, between distribution and bioactivity of those peptides.

Pharmacologically active peptides are located in the venom of sea anemones, which is contained in microscopic organelles known as cnidae. Cnidae are distributed throughout the ectodermal tissue, and each type of cnidae performs a specific biological function: prey capture, aggression or adherence. In turn, each discrete morphological region of a sea anemone, e.g. tentacles, acrorhagi and actinopharynx (throat), has a unique complement of cnidae. We hypothesised that significant morphological regions may have a unique peptide profile owing to the cnidae contained therein and the biological function associated with each region.

Initial proof-of-concept peptidomics, using whole tissue preparations for five discrete morphological regions of *Oulactis* sp., showed that each region has a unique peptide profile. Using MALDI-IMS we examined the same Regions of Interest (ROI's) in *Oulactis muscosa*. Average spectra for each ROI were again shown to be unique. Imaging allowed us to examine individual masses from ROI's; some peptide masses were found ubiquitously throughout the tissue, whilst others were restricted to discrete ROI's.[2] MALDI-IMS data has allowed us to infer biological function of peptides based on tissue distribution, whilst providing us with new regions of interest to examine for their venom components in conjunction with transcriptomics/peptidomics.

1. Chandy, K. G. & Norton, R. S. Peptide blockers of Kv1.3 channels in T cells as therapeutics for autoimmune disease. Curr. Opin. Chem. Biol. 38, 97–107 (2017).
2. Michela L. Mitchell et. al. The Use of Imaging Mass Spectrometry to Study Peptide Toxin Distribution in Australian Sea Anemones. Aust. J. Chem. 70, 1235—1237 (2017).

Applying proteomics to deliver superior and safe plant-based food products

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The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA), eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid (EPA-20:5ω3; DPA-22:5ω3; DHA-22:6ω3) are widely recognised as beneficial to human health. Through metabolic engineering seven fatty acid desaturases and elongases were introduced into canola to convert oleic acid (OA) to DHA in a single pathway expression vector. For genetically modified products, food/feed and environmental risk assessment is required and involves evaluation of each transgenic protein, including protein stability and plant expression levels. Mass spectrometry-based proteomics was employed to demonstrate the gastrointestinal stability of the seven ω3LCPUFA enzymes. Current antibodies, have not proved useful in the characterization of these enzymes because of tight membrane association and non-specific cross-reactions. Therefore, we developed LC-MS/MS based methods to evaluate these transgenic membrane proteins. The proteins were digested with pepsin under simulated gastric fluid conditions for up to 60 min, followed by complete trypsin digestion. The decline of tryptic peptides was used as a proxy for intact protein, and the appearance of peptic peptides indicated the in vitro digestibility of the transgenic proteins. We applied a similar principle to quantify each target protein in different plant tissues by LC-MS/MS. The level of tryptic peptide markers, in 250 µg of total protein, was quantified using a spiked internal standard. The results from an example study demonstrated that >80% of the full-length protein was digested within 10 or >93% after 60 min of incubation. Applying the LC-MS/MS method in transgenic plants; we demonstrate that seed-specific promoters correctly regulated expression of transgenes only in developing and mature seed, and that the enzymes were present at low levels (ng per mg). By examining specific peptides (unique to the targets), this approach provides highly selective and sensitive measurement of membrane proteins. The LC-MS/MS methods described here are applicable to food/feed and environmental safety assessment.

Analysis of phenotypic variation in canine plasma metabolites

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In the last few centuries, the domestic dog has undergone selection for specific traits and breed diversification, forming genetically isolated populations. It is evident that certain breeds are predisposed to simple Mendelian disorders and complex diseases such as cancer. Phenotypic variation in metabolite levels in plasma will vary with genotype, and may reflect susceptibility to disease. Therefore, analysis of the source and extent of variation will potentially be useful in disease investigation and biomarker development. The aim of this study was to characterise the natural metabolite variation and identify genes involved in regulating metabolite abundance in healthy German Shepherd dogs. Using the Illumina Canine HD array, 82 healthy German Shepherd dogs were genotyped across over 172,000 markers. Plasma metabolites, including amino acids, sugars, and fatty acids, of these dogs were quantified using targeted liquid chromatography-mass spectrometry. Genome-wide association analyses of plasma metabolites were conducted to examine genetic influences on metabolite variation.

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Discovery and characterisation of a novel human lysine methyltransferase by CRISPR/Cas9 knock out and SILAC proteomics

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Lysine methylation is widespread on human proteins, however the enzymes that catalyse its addition remain largely unknown. This limits our capacity to study the function and regulation of this modification. We used the CRISPR/Cas9 system to knock out putative protein methyltransferases *METTL21B* and *METTL23* in K562 cells, to determine if they methylate translation elongation factor 1A (eEF1A). The known eEF1A methyltransferase *EEF1AKMT1* was also knocked out as a control. Targeted mass spectrometry revealed the loss of lysine 165 methylation upon knock out of *METTL21B*, and the expected loss of lysine 79 methylation on knock out of *EEF1AKMT1*. No loss of eEF1A methylation was seen in the *METTL23* knock out. Recombinant METTL21B was shown *in vitro* to catalyse methylation on lysine 165 in eEF1A, confirming it as the methyltransferase responsible for this methylation site. To gain insight into the specific function of METTL21B-mediated methylation of lysine 165 in eEF1A, we used SILAC followed by LC-MS/MS to analyse the proteomes of two different *METTL21B* knock outs and compare them to wild-type K562 cells. Two different knock outs of *EEF1AKMT1* were also analysed for proteomic changes and were compared to wild-type K562 to determine the effects of loss of lysine 79 methylation. This revealed specific upregulation of large ribosomal subunit proteins upon *METTL21B* knock out, which was not seen upon *EEF1AKMT1* knock out, and changes to further processes related to eEF1A function in knock outs of both *METTL21B* and *EEF1AKMT1*. This indicates that the methylation of lysine 165 in human eEF1A has a very specific role in translation.

1. Hamey, J. J., Wienert, B., Quinlan, K. G., & Wilkins, M. R. (2017). METTL21B is a novel human lysine methyltransferase of translation elongation factor 1A: discovery by CRISPR/Cas9 knock out. Molecular & Cellular Proteomics, mcp-M116.

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Bioinformatics analysis of DIA based mass spectrometry data to quantify the protein expression

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Quantification of proteins is one of the fundamental applications of proteomics, and has the potential to yield insights into the biology of organisms, especially the response to changes in the internal or external environment. Mass spectrometry-based protein quantification (label-based and label-free) has become a widely used approach to measure relative and absolute abundances of proteins, and has been applied for the discovery of disease-specific biomarkers for early diagnosis and prognosis [1]. In this study, the generalized protocol for the quantification of SWATH-MS data is presented using Skyline software [2]. SWATH-MS (Sequential Window Acquisition of all THeoretical fragment ion spectra coupled to Tandem Mass Spectrometry) is a novel quantification technique that operates in data-independent acquisition (DIA) mode and helps in recording all the peptides' precursors present in the biological sample, including low abundant proteins (such as, plasma proteins). In this protocol, the key steps of quantitative analysis, such as ion chromatogram extraction and, quantification at peptide and protein levels have been presented using publically available dataset. The expression levels of peptides and proteins have been statistically evaluated to identify up and down-regulated proteins. This protocol has the potential to be used to measure the abundances and expressions of peptides extracted from different cancerous samples for the discovery of novel biomarkers. However, such an application shall be undertaken in a future study.

1. Sethi, M.K., Thaysen-Andersen, M., Kim, H., Park, C.K., Baker, M.S., Packer, N.H., Paik, Y.K., Hancock, W.S. and Fanayan, S., 2015. Quantitative proteomic analysis of paired colorectal cancer and non-tumorigenic tissues reveals signature proteins and perturbed pathways involved in CRC progression and metastasis. Journal of Proteomics, 126, pp.54-67.
2. Noor Z, Adhikari S, Mohamedali A, Ranganathan S. Quantification of Proteins from Proteomic Analysis. Encyclopedia of Bioinformatics and Computational Biology, Elsevier, 2017, accepted.

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Phosphoproteomic screening of exercise mimetics reveals drug interactions and identifies PAK2 as a regulator of glucose metabolism

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Exercise improves health through adaptive metabolic and mechanical remodelling, mediated by a network of kinases in response to homeostatic stress. The phosphoproteome of acutely exercised human skeletal muscle provides a blueprint of kinase regulation to promote health. However, of this phosphoproteome, only 10% of the >1000 regulated phosphosites have a known upstream kinase. Consequently, an *in vitro* model of exercise is needed to further characterise the role of known and novel kinases. We therefore performed a phosphoproteomic screen of 10 candidate exercise-like treatments in rat L6 myotubes quantifying 20,249 unique Class I phosphopeptides, of which 37.8% were regulated in at least one treatment. Of the regulated phosphosites in exercised human skeletal muscle that mapped to the *in vitro* exercise mimetics, 74% were regulated by at least one treatment. We predicted the combination of isoproterenol and thapsigargin to most closely recapitulate the exercise phosphoproteome. As exercise involves multiple stimuli, we measured the phosphoproteome of this combination to determine the extent of interactions between exercise-like treatments. Synergy between the treatments produced a phosphoproteome closer to exercise than either treatment or their expected combination, revealing the importance of interactions between stimuli.

Our phosphoproteomic data was also used to predict the involvement of >20 kinases not previously implicated in exercise signalling that may be attractive drug targets. One of these targets was PAK2, previously implicated in cytoskeletal dynamics, however its function in muscle is incompletely understood. CRISPR/Cas9 knockout of PAK2 in L6 myotubes revealed roles as a negative regulator of glucose uptake via an increase in plasma-membrane localised GLUT4. Phosphoproteomic screening identified several novel PAK2 substrates enriched in vesicle trafficking and endosomal recycling. Our data suggests that PAK2 inhibition is a potential novel strategy to promote glucose uptake into muscle cells relevant for the treatment of hyperglycemia and type-2 diabetes.

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Sampling ovarian cancer for proteomic analysis

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Extensive research into the genomic and transcriptomic profiles of cancer has identified important biomarkers of prognosis. High-throughput profiling of the cancer proteome will give us further insight into factors that drive the malignant phenotype and underlie treatment response and resistance in individual ovarian cancer (OC) sub-types. The feasibility of reliable proteomics analysis of tissues has been enabled by using Pressure Cycling Technology (PCT) to digest small tissue samples, followed by SWATH-MS. Advantages of SWATH-MS include favourable sensitivity and reproducibility comparable to targeted proteomics, but without the need to optimise an assay prior to data collection.

Cancer tissues are complex specimens and some tumours, including OC, can be large. An important requirement for comparative proteomic analysis is that the effect of tissue composition and sub-sampling is understood. Therefore, the aims of this study are to i) establish a pre-analytical workflow for PCT-SWATH-MS analysis of OC and ii) investigate the inter- and intra-individual proteomic variability in multiple sub-samples from primary and matched metastatic disease.

Eleven high-grade serous OC cases were identified for this pilot study through the Gynaecology Oncology Biobank at Westmead Hospital. To assess tissue content, each specimen was cryo-sectioned and stained with haematoxylin and eosin (H&E). Two to five areas with varying cancer and stromal content were cored from frozen tissue (CryoXtract CXT350). A second H&E stained section was taken to confirm the cored areas. The cores were transected to give smaller segments (4-11 segments per core). The median wet weight of the samples was 2.5 mg (ranging from 0.4-6.3 mg). A total of 469 sub-samples from 21 matched primary and metastatic specimens were prepared for PCT-SWATH-MS.

A pre-analytical workflow for coring frozen ovarian tumours has been established and methods have been optimised for sample processing. A strategy was developed to reduce potential batch effects and samples analysed by SWATH-MS.

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The good (beer), the bad (hydrolysis) and the evil (gluten) - Using LC-MS to uncover the hidden gluten in craft beers.

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Coeliac disease is a condition that affects about one percent of the population, while non coeliac gluten sensitivity may affect up to a further 10% of the population. There is no current treatment for either of these conditions other than strict adherence to a life-long gluten-free diet. The current methods for measurement involve the use of antibody-based techniques including ELISA, but these approaches have some shortcomings. The use of ELISA to measure hydrolysed gluten, as present in fermented products such as beer, is a topic of hot debate. Liquid chromatography mass spectrometry (LC-MS) analysis has been introduced with an aim to develop quantitative markers for detection of the presence/absence and quantification of gluten in a diverse range of food matrices. We present our recent findings using LC-MS to detect hydrolysed gluten in gluten-free and gluten-reduced beers that are labelled as <20 ppm as judged by ELISA. LC-MS analyses revealed the presence of gluten peptides derived from hydrolysed gluten fragments, many of which were >30 kDa in size. Peptides representing all classes of hordein were detected in the conventional beers, but also alarmingly in several of the gluten-reduced beers. In a barley-based beer brewed from an ultra-low-gluten variety of barley, only a single gluten isoform known to be present was detected. The presence of large protein fragments in the gluten-reduced beers after prolyl endoprotease (PEP) treatment and/or proprietary processing are a cause for concern for those people with Coeliac disease as they may contain immunopathogenic sequences that could elicit adverse reactions.

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Top-down proteomics investigation of age gelation mechanisms in genetically selected milks

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Genetic variation of milk proteins produces milks with vastly different functionalities. For example, it is known that milk composed of A κ-casein and A2 β-casein is less likely to form a rennet gel, the first step in the cheese making process. In this experiment twelve milk groups were selected with specific κ- and β-casein and β-lactoglobulin variants to determine if a particular combination of milk proteins provides a more shelf-stable UHT skim milk. Another aim was to investigate the mechanism/s of age gelation on milks controlled for protein variation. Out of the twelve milk groups, group 9 (AB κ-CN, A1A2 β-CN and AB β-Lg) and group 11 (AB κ-CN, A2A2 β-CN and AB β-Lg) suffered from age gelation over the storage time of nine months, although this was not attributed to the milk protein genetic variants. Top-down mass spectrometry analyses identified 209 protein compounds and numerous post-translational modifications, including 58 intact proteoforms and 151 degradation products. UHT treatment induced lactosylation of the most abundant proteins. All twelve groups of milk suffered from proteolytic degradation during storage. Overall 47 peptides were positively associated with high viscosity; 34 (72%) resulted from β-casein proteolysis, and 20 of these (59%) had cleavage sites previously identified from the extracellular heat stable protease AprX from *Pseudomonas*. Microbial population analysis of the raw milks prior to UHT treatment showed several bacterial taxa lacking in the two groups that suffered from age gelation. Age gelation in these milks could have been initiated by specific proteolytic degradation and a lower microbial diversity may have also contributed to this mechanism.

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A Novel Data Independent Acquisition Method for Hemoglobin Variant Identification in Clinical Research

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Inherited hemoglobin disorders are common worldwide due to population migration. These disorders can be characterised by mutations in the globin gene which may result in a structural abnormality or a reduction in the rate of synthesis of one of the globin chains. A structural change may be harmless, however, some may impair function of hemoglobin and oxygen transport. Here, we will describe a SONAR rapid quadrupole scanning data independent acquisition (DIA) method for the precise identification of several variants that may be encountered in either the α- or β-hemoglobin chains.

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Dietary supplementation of whole dried sugarcane (WDS) in a high fat diet mouse model modulates liver inflammatory pathways

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Chronic inflammation involves dysregulation in the synthesis of pro-inflammatory mediators which are associated with several diseases such as obesity and diabetes. Some nutraceuticals are known to possess anti-inflammatory properties based on their high content of phytochemicals and fibres. We have previously demonstrated that ethanol extracts of whole dried sugarcane (WDS) regulate the expression of crucial inflammatory mediators *in vitro* in an LPS induced colon cancer cell model. In the current study, we evaluated the effects of WDS in a high fat (HF)-fed mouse model by employing multiplex ELISA and SWATH-based proteomics studies to examine cytokines and liver protein expression, respectively. Benefiber® (BF), a commercially available

food supplement, was also examined as an alternative fibre source. Dietary supplementation using either WDS or BF produced any significant shifts in pre-diabetic markers such as weight gain or glucose tolerance tests. Inflammatory and diabetes markers in plasma were not altered in the presence of these supplements. However, protein expression from 2,388 proteins quantified across 40 samples in liver tissue showed that WDS significantly repressed the inflammatory modulator STAT3 and the selenium-associated proteins SEP15 and SECS compared to the HF group, events not observed after BF supplementation. Ingenuity Pathway analysis from this data predicted WDS would repress hepatic functions such as incidence of liver tumour, fibrosis of liver, and liver metastasis. These results suggest that the nutraceutical properties of WDS might be useful in modulating inflammatory properties of the liver and warrant exploration in other disease models.

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Detecting protein-protein crosslinks formed during food processing

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During food processing and storage, insult-induced crosslinks form between proteins, which profoundly influence the nutritional value and properties of food. One crosslink that forms with heating and basic pH, two typical insults used in food preparation and processing, is lysinoalanine. This crosslink has been shown to decrease levels of lysine, an essential amino acid, and reduce digestibility. *In vivo* studies suggest that it may be toxic. Despite the adverse effects of lysinoalanine, its formation and biological fate is not well understood. The typically low relative abundance and complex fragmentation pattern of crosslinked peptides compared to linear peptides, together with several other factors, makes investigating crosslinks in peptides challenging. To simplify the complex fragmentation patterns and narrow the potential number of analytes, we subjected two short synthetic peptides, each five amino acids long, to heat and alkaline treatment and identified a crosslink of lysinoalanine between the peptides. Using mass spectrometry we have characterised the fragmentation pattern of the model and identified a diagnostic ion for the lysionalanine crosslink. This diagnostic fragmentation pattern has the potential to be used to identify the location of lysinoalanine formation in proteins, which is not presently possible through standard or customised bioinformatics software. Knowing where lysinoalanine forms enables this structural aspect to be included in digestion models. These results also give clues to help us predict how altering food preparation procedures will influence formation of lysinoalanine and the opportunity to increase foods' nutritional value.

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Charting the tumour suppressive signaling networks of CHK kinase in colorectal cancer cells with a novel tyrosine-specific phosphoproteomics approach

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Overactivation of Src is involved in tumorigenesis and metastasis of numerous cancer types, including colorectal cancer[1]. Src is over-activated in >80% of colorectal cancers (CRC) and is involved in the progression of CRC.

Oncogenic mutations of Src are rare, implying that overactivation of Src in cancer cells is caused by dysregulation of its upstream regulators. CSK homologous kinase (CHK) is an upstream inhibitor of Src-family kinases (SFK)[2]. CHK is expressed in normal mouse colon samples but significantly decreased in CRC cell lines[3-4]. We recently demonstrated that expression of recombinant CHK in CRC cells had significant impact on the anchorage independent growth and invasiveness of the cells, suggesting CHK as a potential CRC tumour suppressor[4]. As a protein tyrosine kinase, CHK exerts its tumour suppressive action by phosphorylating and inhibiting Src as well as phosphorylating other non-SFK proteins.

We aim to use quantitative phosphoproteomics approaches to define the signaling mechanisms and identify direct substrates of CHK. We have completed the analysis of the changes in phosphoproteome induced by CHK in CRC cells using conventional global phosphoproteomics by Ti^{4+} based-IMAX[5-7]. Although this approach reveals the involvement of many serine/threonine kinases in the tumour suppressive action of CHK, it failed to provide sufficient information and depth to characterise the global changes in phosphotyrosine proteome induced by CHK expression.

Therefore, we aim to utilize triple-substitution mutants of SH2 domains, termed SH2 superbinders due to their markedly increased affinity to phosphotyrosine residues, to define the changes in phosphotyrosine proteome of CRC cells induced by CHK expression[8-10]. Specifically, SH2 superbinders will be used to enrich phosphotyrosine-containing tryptic peptides prior to their identification and quantitation by sophisticated tandem mass spectrometry approach. These two different approaches will allow us to comprehensively identify CHK substrates and define the signaling networks governing its tumour suppressive action in CRC cells.

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The reduced protein methylation network in the early-branching protozoan parasite, *Giardia duodenalis*

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Giardia duodenalis causes over 300 million cases of gastroenteritis (giardiasis) annually, and has unique cell biology shaped by its early-branching origins in the eukaryotic phylogeny. The methylation network in *Giardia* is highly reduced relative to other eukaryotes, lacking canonical arginine protein methyltransferases or demethylases and seemingly confined to six SET-like lysine protein methyltransferases. We undertook the first efforts to characterize methylation in *Giardia* and confirmed this unusal methylation profile in multiple seminal lines via immunoblotting, detecting mono-, di- and tri-methylation of lysine, but no arginine methylation of any known type. Immunoaffinity enrichment and mass spectrometry of lysine methylation in the infective stage (trophozoite) identified 492 methylation sites on 304 proteins, with the majority detected as mono-methylated (84.76%). Many proteins associated with the *Giardia* cytoskeleton, which is involved in adhesion, motility and virulence, were detected as methylated, including microtubule, motor, axoneme-associated and annexin-repeat proteins. We detected methylated helicases, ribosomal/ribonucleoproteins and histone H2 and H3 variants, suggesting a role for methylation in gene regulation, which is implicated in drug resistance. Preliminary inhibitor screening has demonstrated the methyltransferase inhibitor Chaetocin, and related idithiodiketopiperazine (ETP) analogues, inhibit trophozoites, though with lower potency than recorded in mammalian lines. Our future work aims to explore the sites and substrates of methylation in the transmissive life-stage (cyst), and the effect of methylation inhibitors on encystation (differentiation) and drug resistance.

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Peptidomics and metabolomics reveal the role of cysteine, aspartic and metallo proteases in the haemoglobin digestion pathway of the malaria parasite.

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The malaria parasite requires amino acids for protein synthesis and a major source of amino acids is via the digestion of host haemoglobin. Digestion of haemoglobin occurs within a specialised food vacuole via a semi-ordered proteolytic cascade that is mediated by different protease including aspartic, cysteine and metallo proteases. As haemoglobin digestion is essential for parasite survival within the red blood cell, understanding the exact interplay between the different classes of proteases within the food vacuole is crucial. In this study, we have developed combined metabolomics and modified proteomics workflows to identify the 'signature peptide' libraries that are generated as a result of specific inhibition of each protease class involved in haemoglobin digestion. The combined peptidomics analysis revealed that inhibition of specific proteases resulted in unique changes in the abundance of endogenous peptides. The results obtained were highly sensitive and could distinguish between different clans of the same class of protease. For example, specific inhibition of the metallo-aminopeptidases could distinguish unique peptide signatures between different types of aminopeptidases. Inhibition of the clan MA, M1 aminopeptidase resulted in accumulation of peptides containing basic residues, while specific inhibition of the clan MF, M17 aminopeptidase resulted in accumulation of hydrophobic peptides. This difference in selectivity is consistent with previously published *in vitro* studies of substrate specificity. In conclusion, this multi-platform approach provided an extensive coverage of endogenous peptides liberated during haemoglobin digestion within the parasite, and identified specific peptide signatures associated with inhibition of different classes of proteases involved in haemoglobin digestion. This will subsequently enhance our understanding of the interplay between the different proteases involved in haemoglobin digestion and reveal the mechanisms of action and resistance for drugs that target this pathway.

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Mycoplasma pneumoniae presents proteolytically processed proteins on its cell surface

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Proteolysis is an essential and ubiquitous post-translational protein modification (PTM) for the normal functioning of many biological systems. In prokaryotes, the scale of proteolytic regulation of the proteome is poorly characterised. The first systems-wide analysis of proteolytic cleavage in the bacterium *Mycoplasma hyopneumoniae*, a minimal genome porcine pathogen, was published in 2017. This landmark study demonstrated an unprecedented level of proteolytic processing targeting surface proteins with a role in interactions between *M. hyopneumoniae* and receptors on the surface of target host cells. Mycoplasma species are often highly host-adapted and known to infect vertebrates, invertebrates and plants, utilising specific individual characteristics and virulence factors.

To expand our knowledge of proteolytic processing, we examined the systems-wide processing events in the human respiratory pathogen *Mycoplasma pneumoniae* using an N-terminome approach. This method used dimethyl labelling on the protein level and mass spectrometry to identify cleavage sites in 391 proteins. These data indicate that 56% of the predicted proteome is affected by proteolytic processing, producing many novel proteoforms from a genome of 688 ORFs. Biotinylation and trypsin shaving of cell surface proteins was used to identify 160 proteins located on the surface of *M. pneumoniae* and over 80% of these (134 proteins) are targeted by proteolysis. Affinity chromatography was used to characterise putative host-pathogen protein interactions, demonstrating that surface proteins which undergo more extensive processing were correlated with a greater variety of interactions with host molecules. Notably, these data mirror the findings reported in *M. hyopneumoniae,* as well as a recent study of *Spiroplasma citri*, which demonstrates that proteolytic processing may be a fundamental mechanism required to generate

proteoforms on the surface of the Mollicutes. These findings have significant implications for our understanding of protein maturation and the development of vaccines against these important pathogens.

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Monitoring of yeast health using MALDI-TOF MS

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Beer is the third most consumed beverage after coffee and tea, and therefore relies on strict quality assurance procedures to ensure consistent, high quality beer and consumer satisfaction. During brewing, yeast are exposed to a variety of stressors and must be able to respond to fluctuations in the environment such as pH, ethanol concentration and nutrient content. The re-use of yeast in subsequent fermentations (known as serial repitching) and their ability to maintain viability and vitality is also important for brewing performance. Recently, the applicability of molecular profiling using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with Biotyper software was investigated for identification of beer spoilage microorganisms from routine biological quality control samples in a South Australian brewery. To expand the Biotyper platform and allow more comprehensive quality control practices, we assessed the monitoring of yeast fermentation performance using MALDI-TOF MS and incorporation a specific yeast health library into the database. Reference mass spectrum profiles of lager yeast (*Saccharomyces pastorianus)* exposed to osmotic, oxidative, ethanol, cold and nutrient stress were established and integrated into the Biotyper library. The applicability of monitoring yeast health was assessed by testing samples obtained from key points in production from a local South Australian brewery and the proteome analysed. Proteins that differentiate these stressed states from healthy yeast were subsequently identified using liquid chromatography tandem-mass spectrometry (LC-MS/MS). This technology will aid in improving brewing production, reducing economic waste and accelerate the growth of the industry.

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Lazyprot: a comprehensive software for the bioinformatic processing and pre-processing of proteomic data

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In recent years, the number of technologies and instruments available and the field of applications for mass spectrometry have grown exponentially and with it the need for application software able to process with this tremendous amount of data generated. In parallel, the latest effort pulled by industry to make mass spectrometry affordable and the interface usable even by neophytes have widen the range of users and related knowledge about the omics techniques.

When it comes to data processing, often considered a bottle neck of many experiments, new tools for the analysis and interpretation of data are released regularly by industry and the scientific community [1, 2], however a regularly forgotten side of it is the pre- and post-processing of the data to be inputted or outputted by such elaborated pipelines. To format correctly or extract useful information from omics analysis sometime the best option remains bioinformatics [3].

Without a proper support bioinformatics can very quickly become frustrating and requires a substantial time and effort investment before becoming useful. To overcome this, Lazyprot was designed to provide a comprehensive and versatile solution to common problems when dealing with bioinformatics data but also attempt to provide innovative tools for the processing of proteomics quantitation and more specifically the SWATH workflow.

Generation of proportionated venn diagrams, formatting and combination of fasta files, this tool also provides some solutions for mzML spectra representation and visual de novo sequencing as well as in silico Library generation for SWATH experiments and liquid chromatography retention time transposition from one system to another. All these tools are packaged in a Python language interface without dependencies or installation needed to make proteomic analysis as easy as possible.

1. Deutsch EW, Mendoza L, Shteynberg D, et al. A Guided Tour of the Trans-Proteomic Pipeline. Proteomics. 2010;10(6):1150-1159. doi:10.1002/pmic.200900375.
2. MacLean B, Tomazela DM, Shulman N, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010;26(7):966-968. doi:10.1093/bioinformatics/btq054.
3. Cappadona S, Baker PR, Cutillas PR, Heck AJR, van Breukelen B. Current challenges in software solutions for mass spectrometry-based quantitative proteomics. Amino Acids. 2012;43(3):1087-1108. doi:10.1007/s00726-012-1289-8.

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Unravelling the connection between Salmonella and Ankylosing Spondylitis

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The association of HLA-B27 with Ankylosing Spondylitis (AS) is one of the strongest associations between a HLA molecule and an autoimmune disease. This association can, in part, be explained by the atypical characteristics of HLA-B27 to form homodimers, which act through non-classical pathways and trigger aberrant immune responses. Various cellular stresses have been proposed to induce homodimer formation, and here we show that infection by *Salmonella typhimurium* can also induce homodimer formation. Considering that AS patients often report developing disease post infection with gastrointestinal bacteria and as a sequela of infection induced reactive arthritis, HLA-B27 homodimers could play role in disease pathogenesis. The aim of this study is to understand the cellular requirements for homodimer formation and to provide insights on how these homodimer species trigger the immune system.

Methods: Differences in the proteome between *Salmonella typhimurium* infected and uninfected cells were analysed by data dependent acquisition mass spectrometry. Significantly regulated proteins were identified and further analysed.

Results: Our analysis identified 122 upregulated and 533 downregulated proteins post wild-type *Salmonella typhimurium* infection. These include proteins involved in the MHC presentation pathway, oxidative phosphorylation, glutathione metabolism, cell cycle and apoptosis. Proteins in the toll-like receptor signalling pathway, TRIF and part of NF-kb signalling pathway, which are generally involved in bacterial detection and signalling, were also observed to be modulated. The pathways important for homodimer formation have subsequently been interrogated using pharmacological inhibition and using mutant *Salmonella typhimurium* strains.

Conclusion: This study provides insights on how homodimers are formed and their potential mode of action in disease. Consequently, opening the doorway for designing targeted therapies for Ankylosing Spondylitis.

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Towards the generation of a comprehensive proteome map for phytophthora cinnamomi, the causal agent of native dieback

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Phytophthora cinnamomi is a pathogenic oomycete that poses a significant threat to global biodiversity. It causes tree death and loss of vegetation/ crops by attaching to the fine-feeder roots and releasing apoplastic and cytosolic effectors. The destructive nature of these effectors during plant-pathogen interactions drives a complex array of defense responses and when successful, the pathogen causes plant death. *Phytophthora cinnamomi* exhibits various life stages, which enable it to survive through harsh environments. This has contributed to its success as a pathogen as it takes advantage of weakened potential hosts.

However, the molecular mechanism of *P. cinnamomi* pathogenicity is not well-understood. In order to better understand the pathogen, a proteomic approach was used to dissect sub-cellular proteomes of *P. cinnamomi*. We were able to extract high quality intracellular and secreted proteins of *P. cinnamomi* and perform preliminary analysis of the intracellular proteome using gel-free shotgun proteomics. We identified 612 unique proteins when the mass spectrum was matched against the *P. cinnamomi* genome. From here, a 2-dimensional separation by reverse phase chromatography and liquid chromatography mass spectrometry will be developed to analyse the intracellular and secreted proteomes. We anticipate this approach will lead to a greater level of protein identification and provide a comprehensive biochemical snapshot of the pathogen.

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A Comparative Qualitative and Quantitative Assessment of SONAR for High-Throughput Proteomic Applications

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The continued requirement to analyze larger sample cohorts to detect quantitative biologically significant differences is becoming of greater importance and placing greater demands on valuable instrument time. Traditionally, proteomic LC-MS analyses have been conducted using nanoscale chromatography in combination with data dependent analysis (DDA). However, the adoption of faster chromatography to increase sample throughput and data independent approaches (DIA) to overcome some of the shortcomings of DDA are proving increasingly popular. A number of DIA strategies with enhanced specificity exist, such as SWATH, whereby the quadrupole is stepped across a mass range of interest to increase specificity. This however can have drawbacks when utilizing faster chromatographic methods, since the duty cycle of the instrument is challenged. An alternate DIA method which also uses the quadrupole for selectivity is SONAR, whereby the quadrupole is scanned as opposed to stepping over the mass range of interest. SWATH and SONAR data were collected for a K562 cell line, demonstrating the importance of speed and specificity for accurate quantification and improved qualitative performance. When short chromatographic runs are implemented (i.e. 30 mins) a higher number of protein groups were identified for SONAR. Comparatively, 600 additional proteins were identified for SONAR when compared with SWATH. Quantitaitve gains are also observed for SONAR data showing high numbers of data points across chromatographic peak widths (typically 3 sec at half height), whilst maintaining optimum S/N. The enhanced specificity provided by the technique also demonstrates non-interfered detection of fragment ions.

Overall, results from this comparative study highlight the advantages of SONAR over SWATH based DIA methods. The implementation of a scanning quadrupole with SONAR acquisitions allow data to be acquired at a faster rate, therefore providing a higher number of protein identifications with higher quantitative precision, particularly for high throughput analyses when using microflow chromatography for example.

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Evaluating paper spray ionization and dried plasma spot analysis techniques for quantitation of the prostate cancer drug abiraterone

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Abiraterone acetate is administered as a prodrug to metastatic, castration-resistant prostate cancer (mCRPC) patients to prolong their survival. Abiraterone acetate is readily metabolized into potent Androgen Receptor inhibitors Abiraterone and Abiraterone D4A (D4A). Due to reported pharmacokinetic (PK) variability of Abiraterone, it is a potential candidate for therapeutic drug monitoring (TDM). Here, we developed and validated a bioanalytical method as per the US FDA guidelines for the absolute quantitation of Abiraterone and D4A in patient plasma using ultra-high performance liquid chromatography (UHPLC) coupled with high-resolution accurate mass (HRAM) mass spectrometer (MS). The quantitation was linear for the range of 0.074–509.6 ng/mL for Abiraterone and 0.075–59.9 ng/mL for D4A. Steady-state trough level plasma samples from two-time points of seventeen mCRPC patients were quantified using the assay.

We explored using this assay with paper spray ionization (PSI) and dried plasma spot (DPS) techniques. In these techniques, a small volume (6 µL in PSI and 20 µL in DPS) of patient plasma was spotted on a paper substrate and was dried for 2 hours under ambient conditions. In case of PSI technique, the samples were introduced directly into HRAM MS for quantitative analysis by applying voltage and solvent on the paper substrate. For DPS technique, the samples were processed to recover the drug and then were quantitatively analyzed by LC-MS. The range of quantitative values reported for Abiraterone and D4A in mCRPC patient samples analyzed by conventional liquid plasma method were from 2.8–26.2 ng/mL and 0.26–2.6 ng/mL respectively whereas the corresponding values from PSI technique were 4–5 times higher. The artificially high levels of quantitative values in PSI technique can be attributed to high background interfering ions. The DPS technique needs further exploration given the inherent advantage of low sample volumes involved, ease of sample collection, convenient storage, simple handling and transportation.

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Bioarchaeological Proteomics: Development and application of Sample Preparation Methodology for Analysis of Ancient Egyptian Human Skin Samples

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Proteins are more resilient molecules than DNA, which means there are certain advantages in analysing ancient proteins rather than ancient DNA. Shotgun proteomics analysis involves the characterisation of the protein profile of a given biological sample using nano flow liquid chromatography – tandem mass spectrometry, which generates large scale data from miniscule amounts of material. Archaeological research is often limited to tiny amounts of very precious samples available for scientific analysis. However, there is no validated proteomics workflow in the published literature that could be applied to ancient human skin tissue samples.

We present here details of our work on the optimization and application of sample preparation methods for shotgun proteomics profiling analysis of ancient skin samples, by first using modern human skin tissue as a surrogate. We have tested two different grinding techniques, glass bead grinding and liquid nitrogen grinding, for use in the process of protein extraction. We have also tested two different protein digestion techniques, SDS-PAGE and in-gel digestion, and filter-aided sample preparation (FASP) with in-solution digestion.

Using an optimized approach, we have subsequently performed a shotgun proteomics analysis to investigate the protein profile of a set of four ancient human skin samples. These were sampled from an ancient Egyptian child in funerary basket of the Old Kingdom (2200 – 2000 BC). The results of these analyses are quite distinct from those of our previous work on skin samples from ancient Egyptian mummies. In addition to the immune response and inflammation related protein is, we identified numerous proteins related to skin infections. Thus, we have been able to generate unique archaeological information using molecular analysis techniques, including an indication of the health state of the individual at the time of death.

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Comparison of SDS-PAGE gel slice protein fractionation and reversed-phase spin column peptide fractionation for use in quantitative shotgun proteomics in a variety of biological systems

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One of the most important steps in quantitative shotgun proteomics analysis is fractionation, at either the protein or peptide level. SDS-PAGE fractionation of proteins, followed by in-gel digestion of proteins is a robust approach that has found widespread application in the field, including in our laboratory. In this study, we set out to determine how an orthogonal fractionation approach compared to our standard workflow in terms of numbers of proteins identified, time involved, ease of use, and applicability to a range of different biological sample types.

Using sample material available from a variety of other projects in our laboratory, we have performed quantitative shotgun proteomics analysis of proteins extracted from a range of biological materials including E. coli, yeast, rice leaf, eucalyptus leaf, human skin and oyster gills. Samples were first analysed using our standard workflow of three replicates of SDS-PAGE fractionation of extracted proteins, followed by in-gel digestion of 16 equal fractions. Samples were then analysed using an alternative workflow involving three replicates of in solution digests of extracted protein using filter assisted sample preparation (FASP), followed by fractionation of peptides into 16 fractions based on hydrophobicity using a reversed-phase spin column. In both cases, peptides were then separated and identified using nanoflow reversed-phase liquid chromatography – tandem mass spectrometry.

Preliminary experiments from analysis of E. coli lysates indicate that the reversed-phase spin column workflow seems to generate more reproducibly identified proteins from the same amount of starting material, and is comparable to the standard workflow in terms of both time involved and ease of use. We will present results from analysis of all the different biological materials involved, including bioinformatic characterisation of the properties of peptides and proteins found to be strongly enriched in one workflow or the other.

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PepWitch: a python module to facilitate FDR-corrected *Benjamini–Hochberg* t-tests by employing same/same analysis on label-free shotgun data.

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Label-free shotgun proteomics results often contain thousands of protein identifications. As such, employing the Student's T-Test to ascertain differences between sample states will invariably introduce false positives. This is termed the multiple testing problem. In the recent past, multiple testing corrections such as the *Bonferroni* or *Benjamini–Hochberg* procedure were employed to account for this noise. *However, these* tests tend to be overly conservative while also eliminating true positive results. In our research, we have developed a new method to help correct the *Benjamini–Hochberg overcorrection through the analysis of the noise inherent within biological replicates. Through a multivariate, non-redundant analysis of six replicates (termed same/same analysis), an internal noise threshold is produced where an appropriate Q value facilitates a one percent false discovery rate. This Q value is then used for subsequent control/treated state comparisons. This research lead to the development of a python script called PepWitch (available soon on github) that can, given at least one state with six replicates, automatically determine the best Q value to use for subsequent control/treated analysis (GPM output only, for now). PepWitch also functions to generate high stringency data based on normalised spectral abundance factors from regular triplet experiments – this is an expansion of the previously available Scrappy module .*

1. Neilson, Karlie A., et al. "Label-free quantitative shotgun proteomics using normalized spectral abundance factors." Proteomics for Biomarker Discovery (2013): 205-222.

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Role of post translationally modified peptides in cancer

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Introduction- A wide variety of normal cellular events including cell signaling, DNA replication and protein degradation are regulated by protein post-translational modification (PTM). Oligopeptides are liberated from proteins during their intracellular degradation via the proteasome and a few of them retain their PTMs and along with native peptides are transported into the endoplasmic reticulum, where they are loaded onto Human Leukocyte Antigen (HLA) class 1 molecules and presented on the surface of antigen presenting cells. The composition of these HLA-peptide complexes dictates T cell recognition and any subsequent immune response. Various studies have shown that in cancer, a dysregulation in different signaling cascades occurs which increases PTMs. The aim of this study is to identify modified proteins and peptides in different hematological cancers including different subtypes of leukemias and myelomas.

Methods- 1 X 10^9 cells of human acute monocytic leukemia cell line, THP-1 (HLA-A*02:01, B*15:11, C*03:03, DRB1*01:01, 15:01, DQB1*05:01, 06:02, DPB1*02:01, 04:02) were lysed. Immunoaffinity purification was used to extract peptides from HLA class I and II alleles. Direct proteome analysis of the peptide ligands was performed using liquid chromatography and tandem mass chromatography (LC-MS/MS).

Results- A total of 5788 peptides were identified from HLA-A*02:01, 2259 peptides from pan HLA class I and 2027 peptides for pan HLA class II. Several different modifications were found; prominently oxidation and deamidation along with more rarely detected phosphorylation (n=37) and methylation (n=100). These rarer modified peptides will be tested in cellular assays, using peripheral blood mononuclear cells hematological cancer patients and healthy donors (as a control group) to determine their ability to evoke a T cell mediated immune response.

Conclusion- This study implicates PTM proteins and peptides as an immunological signature of 'transformed' cancer cells that can lead to development of newer diagnostic tools and better immunotherapeutic approaches.

1. 1. Cobbold M, De La Pena H, Norris A, Polefrone JM, Qian J, English AM, et al. HLA class I-associated phosphopeptides are the targets of memory-like immunity in leukemia. Science translational medicine. 2013;5(203):203ra125.
2. 2. Zarling AL, Polefrone JM, Evans AM, Mikesh LM, Shabanowitz J, Lewis ST, et al. Identification of class I HLA-associated phosphopeptides as targets for cancer immunotherapy. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(40):14889-94.
3. 3. Williamson NA, Rossjohn J, Purcell AW. Tumors reveal their secrets to cytotoxic T cells. Proceedings of the National Academy of Sciences. 2006;103(40):14649-50.

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A blockage, a trickle or a deluge? TAP'ing into viral immune evasion

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ICP47 is an iconic immune modulator encoded by herpes simplex virus (HSV) that hides infected cells from the immune system by inhibiting the presentation of peptides to CD8 T cells. Specifically, ICP47 functions by binding to the transporter associated with antigen presentation (TAP), blocking peptide transport and loading onto MHCI, thus reducing peptide display at the cell surface. During its identification in the 1990s, ICP47 was attributed to have near-absolute species bias for human TAP; however, later work showed that ICP47 can contribute to HSV neurovirulence in mice. Given the continued use of mouse models as a mainstay of HSV research *in vivo*, we re-examined this controversy using a new set of ICP47 null viruses through a combination of infection models and epitope identification and quantification by mass spectrometry. In a mouse flank infection model, deletion of ICP47 did not alter lesion development or virus load, latency, spread and reactivation. By contrast, we show ICP47 does inhibit antigen presentation significantly on HSV-infected mouse cells using *in vitro* antigen presentation assays. Further, we identified more HSV-derived peptides on ICP47-deficient cells than wild-type and subsequently quantified peptide presentation of 13 such epitopes and the impact of ICP47 in cells expressing either mouse or human TAP molecules. These data confirm that ICP47 almost entirely blocks human TAP-mediated peptide presentation, though the degree of inhibit was somewhat peptide-specific; conversely, the effect of ICP47 on mouse TAP was far less profound, resulting in a two- to five-fold reduction in MHC-peptide abundance. These data allow us to put the first numbers on viral immune evasion at the MHC-peptide level and validate the species-specific nature of ICP47. Thus, despite reduced, though nonetheless significant, antigen presentation in mouse cells, ICP47 is not an effective immune modulator in mice where HSV is confined to the peripheral nervous system.

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Label free and TMT proteomic analysis of leaf lamina and leaf growing zones in rice genotypes with contrasting drought tolerance

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Plants require a distinctive cohort of enzymes to coordinate division and the subsequent expansion of cells. Proteomic analysis of ever smaller samples now enables interrogation of the proteome of tissues dissected from the leaf bases of higher plants. Thus, plants exposed to stresses such as drought are amenable to analysis of the proteome. We investigated whether proteins expressed in the mature regions as well as the most immature regions of developing leaves of a drought-tolerant rice landrace (IAC1131) could provide insights into the impact of soil drying on gene expression when compared with the drought-sensitive Nipponbare. Proteins extracted from one-cm long shoot growth zones and the mature leaf lamina were investigated using both Tandem Mass Tags (TMT) and label-free proteomic approaches. While the TMT labelling approach identified more proteins overall than the label free approach, the observed protein abundance trends and biological conclusions reached from both sets of data were in close agreement. The data acquired from both proteomic approaches indicate that the leaf lamina of IAC1131 appears to be better able to cope with stressful conditions by up-regulating a suite of stress and defence response related proteins. Nipponbare, in contrast, lacks the range of stress responses shown by the more stress tolerant variety, and responds to drought stress by initiating a partial shutdown of chlorophyll biosynthesis in an apparent attempt to preserve metabolic resources. While in the leaf growing zone, proteins involved in organization of the meristem and subsequent cell formation were up-regulated in drought in IAC1131, and those proteins involved in oxidation state and response to external stimuli were more likely to be up-regulated by drought in Nipponbare.

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Characterisation of Biotherapeutics Using Native Ion Mobility Mass Spectrometry

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Biopharmaceutical products present a complex analytical challenge to the modern pharma industry. Not only must laboratories characterize the primary structure of protein species, but also the tertiary and quarternary (higher order) structure. One method often used in research laboratories for higher order structure analysis is native ion mobility spectrometry-mass spectrometry (IMS-MS). Due to the expansion of the biopharmaceutical industry and the broadening range of drugs being investigated, instrumentation is required to carry out advanced experiments in a routine manner. We describe a method to carry out native IMS-MS on a routine high resolution LC-IMS-MS platform. We also describe investigations into the applicability of high resolution-IMS-MS (HR-IMS-MS) for biopharmaceutical analysis using a cyclic IMS-enabled research platform.

1. T. W. Knapman, et al. Curr Anal Chem 2013, 9(2), 181-191.
2. T. M. Allison, et al. Nat Commun 2015, 6:8551.
3. C. V. Robinson. Biochem Soc Trans 2017, 45(1), 251-260.

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Consequences of genetic variation and associated proteomes in chronic venous leg ulceration

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

The HFE gene encodes for a cell surface protein which helps to regulate the absorption of dietary iron and its release from storage sites in the body. Two single nucleotide polymorphisms (SNPs) within this gene, p.C282Y and p.H63D, are directly involved in the dysregulation of iron homeostasis, leading to iron overload in various tissues and in some cases, hereditary haemachromatosis. Iron overload in the dermis has been associated with fibrosis and chronic inflammation. We hypothesise that, in combination with chronic venous insufficiency and venous stasis, these mutations in the HFE gene can predispose patients to poorer healing outcomes.

These SNPs were analysed in VLU patients and age matched controls while the protein in patient wound fluid samples was analysed using a SWATH proteomics approach. We found that 17% of the patient cohort (n = 52) had at least one HFE p.C282Y mutant allele, compared to 8% in the control cohort (n = 62). A Pearson's Chi-squared test showed the frequencies of the p.C282Y mutant alleles to be significantly different between the two cohorts ($p = 0.04$).

Correlation of genotype data with the proteomic data, with particular emphasis on proteins related to iron metabolism and tissue integrity, is ongoing. It is anticipated that proteins associated with the HFE gene will have an altered abundance in non-healing or slow healing wounds.

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Human CD52 initiates its immunosuppressive activity via specific sialoforms

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Regulation of T cells is necessary to limit their proliferation and to prevent autoimmune diseases. High expression of the cluster of differentiation protein 52 (CD52) results in suppression of other T cells by interacting with sialic acid-binding immunoglobulin-like lectin receptor-10 (Siglec-10) (1). CD52 is a low molecular weight (1208 Da) glycopeptide with a single *N*-linked, and several potential *O*-linked, glycosylation sites (2). We aimed to perform a comprehensive glycomics/glycoproteomics characterisation of several CD52-Fc recombinant proteins to determine the bioactive glycoforms of CD52. CD52 was expressed as a recombinant protein in fusion with carrier immunoglobulin Fc glycoprotein in host cells (HEK293 or CHO), and was functionally tested for suppression of T-cell proliferation and interferon-γ secretion. *N*-glycans were released after PNGase F treatment and reduced glycans were analysed using porous graphitised carbon-liquid chromatography-MS/MS in (-) mode on an ion trap mass spectrometer. Intact-mass of CD52 analysis was performed by (+) mode reversed phase C8-ESI-MS/MS on a Q-TOF mass spectrometer. The *N*-glycosylation profile of CD52 with the carrier Fc N_{297} glycosylation site mutated showed the presence of several bi-, tri- and tetra-antennary sialylated *N*-glycan structures. This glycan profile was also observed on CD52 after Factor X cleavage from the Fc carrier. *O*-glycan structures were observed by intact-mass analysis of the CD52 glycopeptide after PNGase F treatment, and showed a low abundance of core type-2 di-silaylated structures. Interestingly, the relative abundance of tri- and tetra- antennary sialylated structures, as well as the specific α-2, 3 sialic acid linkage, correlated with two variants of CD52-Fc that showed different immunosuppressive activity. Furthermore, anion exchange chromatography on a Mono Q GL column separated CD52-Fc into glycoforms that varied in their capacity to suppress T-cell proliferation, strongly supporting different sialylation as a determinant of active CD52. These findings define glycan structures responsible for the bioactivity of CD52.

1. 1. Bandala-Sanchez E, Zhang Y, Reinwald S, Dromey JA, Lee B, Qian J, Böhmer RM and Harrison LC (2013) T cell regulation mediated by interaction of soluble CD52 with the inhibitory receptor Siglec-10. Nature Immunol 14: 741-48.
2. 2. Treumann A, Lifely MR, Schneider P and Ferguson MAJ (1995) Primary structure of CD52. J Biol Chem 270: 6088-99.

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Glycosylation features of neutrophilic granules

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Protein glycosylation is important in many processes of the innate immune system. Aberrant glycosylation can modulate the innate immune response including the anti-microbial function of neutrophils. Recently, we discovered a new type of protein *N*-glycosylation, paucimannosidic *N*-glycans ($Man_{1-3}GlcNAc_2Fuc_{0-1}$) on intact bioactive proteins that appear to be significant immune-modulators within the primary granule of neutrophils (Loke *et al.*, *Mol Cell Proteomics,* 2017). To improve our understanding of these glycoepitopes, we here provide a detailed map of the glycosylation of the various granules of human neutrophils. Resting neutrophils from healthy donors were isolated. After gentle plasma membrane disruption, granules were separated by density gradient centrifugation into four fractions containing primary, secondary and tertiary granules, and the secretory vesicles/plasma membrane. The identity of the isolated intact compartments was validated using ELISA of granule-specific markers. Disruption of the granules by sonication, followed by ultracentrifugation, allowed the separation of the luminal and membrane proteins. Released *N*- and *O*-glycans from these proteins were analysed using PGC-LC-ESI-CID-MS/MS. Bottom-up and top-down glycoproteomics experiments were also performed using RP-LC-ESI-MS/MS. In total, 68 *N*-glycans and 9 *O*-glycans were characterised and quantified across the neutrophil granules. Interestingly, granule-specific *N*- and *O*-glycosylation was determined, as illustrated by the significant enrichment of paucimannosidic *N*-glycans and a complete absence of *O*-glycans in primary granules, and very large poly-LacNAc containing bi-antennary complex-type *N*-glycans within secondary granules. Additionally, distinct glycosylation differences were observed between the luminal and membrane proteins indicating differential glycan processing even within the individual granules. Bottom-up and top-down glycoproteomics confirmed that paucimannosylation is a significant glycoepitope within primary granules and that these unusual *N*-glycans are carried by intact proteins. This detailed glyco-map demonstrating peculiar granule-specific glycosylation features of healthy neutrophils is essential to further advance our understanding of granulocytic glycobiology in inflammation and infection.

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Dual function of Ost3 proteins elucidated using glyco- and global proteomics

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N-glycosylation is an essential co- and post- translational modification that affects protein folding, stability and function. *N*-glycosylation is catalyzed by an enzyme complex known as the oligosaccharyltransferase (OTase). Ost3 and Ost6 proteins are subunits of the OTase and found to have different protein substrate binding affinities1. In humans, the orthologues of *OST3* and *OST6* are MagT1 and TUSC3. MagT1 and TUSC3 have been found to transport magnesium as well as assist in glycosylation, hence we hypothesized that Ost3 and or Ost6 proteins could perform similar functions.

Yeast is a model organism where gene manipulation can be used to study protein function. Here, we used a Tet repressible promoter system to achieve a double knockdown/out of *OST3/6* in a yeast strain. Recent advances in mass spectrometry allowed us to study proteins and their modifications extensively. In this study we employed both glyco- and global proteomics to elucidate Ost3/6 function.

We found that Ost3- and Ost6-deficient yeast had a severe growth defect, which was partially rescued by the addition of magnesium. This supports a role for Ost3 proteins in Mg^{2+} transport in yeast, as well as in vertebrates. However, glyco-proteomics did not show any sign of improvement in site specific glycosylation. This result suggested that Ost3 protein-mediated Mg^{2+} transport is independent of glycosylation. Consistent with this, global proteomics revealed extensive changes in ribosomal proteins, metabolic processes and translation. Of these changes, ribosomal proteins were rescued by the addition of excess Mg^{2+}.

The dual function of Ost3 proteins in regulating *N*-glycosylation and in transporting Mg^{2+} appears to be conserved from vertebrates to fungi. Ost3 protein-mediated Mg^{2+} transport is required for normal cellular levels of ribosomes, potentially due to the direct stabilizing effect of Mg^{2+} on intact ribosomes.

1. Polypeptide binding specificities of Saccharomyces cerevisiae oligosaccharyltransferase accessory proteins Ost3p and Ost6p. Jamaluddin, M. Fairuz B., Bailey, Ulla-Maja, Tan, Nikki Y. J., Stark, Anthony P. and Schulz, Benjamin L. (2011) Polypeptide binding specificities of Saccharomyces cerevisiae oligosaccharyltransferase accessory proteins Ost3p and Ost6p. Protein Science, 20 5: 849-855. doi:10.1002/pro.610

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Characterising site-specific *N*-glycosylation facilitated by the oligosaccharyltransferase

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Asparagine (*N*)- linked glycosylation is a common and important post-translational modification (PTM) present in all three domains of life - archaea, eukaryota and bacteria. It has essential roles including facilitating protein folding, stability and function. It specifically refers to the attachment of carbohydrates onto asparagine (Asn) residues, typically within the consensus sequon N-X-S/T, where X cannot be proline. Biosynthesis of the donor glycan substrate for glycosylation is a multi-enzymatic process catalysed by the Alg (asparagine-linked glycosyltransferase) enzymes (Alg1-14). The sequential activity of the Alg enzymes results in a 14-sugar oligosaccharide ($Glc_3Man_9GlcNAc_2$) that is transferred onto a protein acceptor substrate by the oligosaccharyltransferase (OTase). Defects in the *N*-glycan biosynthetic pathway cause changes in glycan occupancy, structure, and changes in protein abundance. While the oligosaccharyltransferase (OTase) is the central enzyme in *N*-glycoprotein biosynthesis, physiological regulation of the enzyme is poorly understood. To test for the presence of a regulatory mechanism controlling OTase activity in response to glycosylation stress, we used SWATH-MS to quantify site-specific changes in glycan occupancy in a yeast model system. We compared site-specific glycosylation in yeast with defects in LLO structure (*alg6Δ*) and abundance (tunicamycin-treated cells). We identified a subset of sites that were inefficiently glycosylated in tunicamycin-treated cells but which remained efficiently glycosylated in *alg6Δ* cells. Our findings suggest that sequence motifs regulate site-specific OTase activity to ensure glycosylation is optimally targeted in conditions of glycosylation stress.

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Carcinoembryonic antigen glycosylation – a highly underestimated cancer marker?

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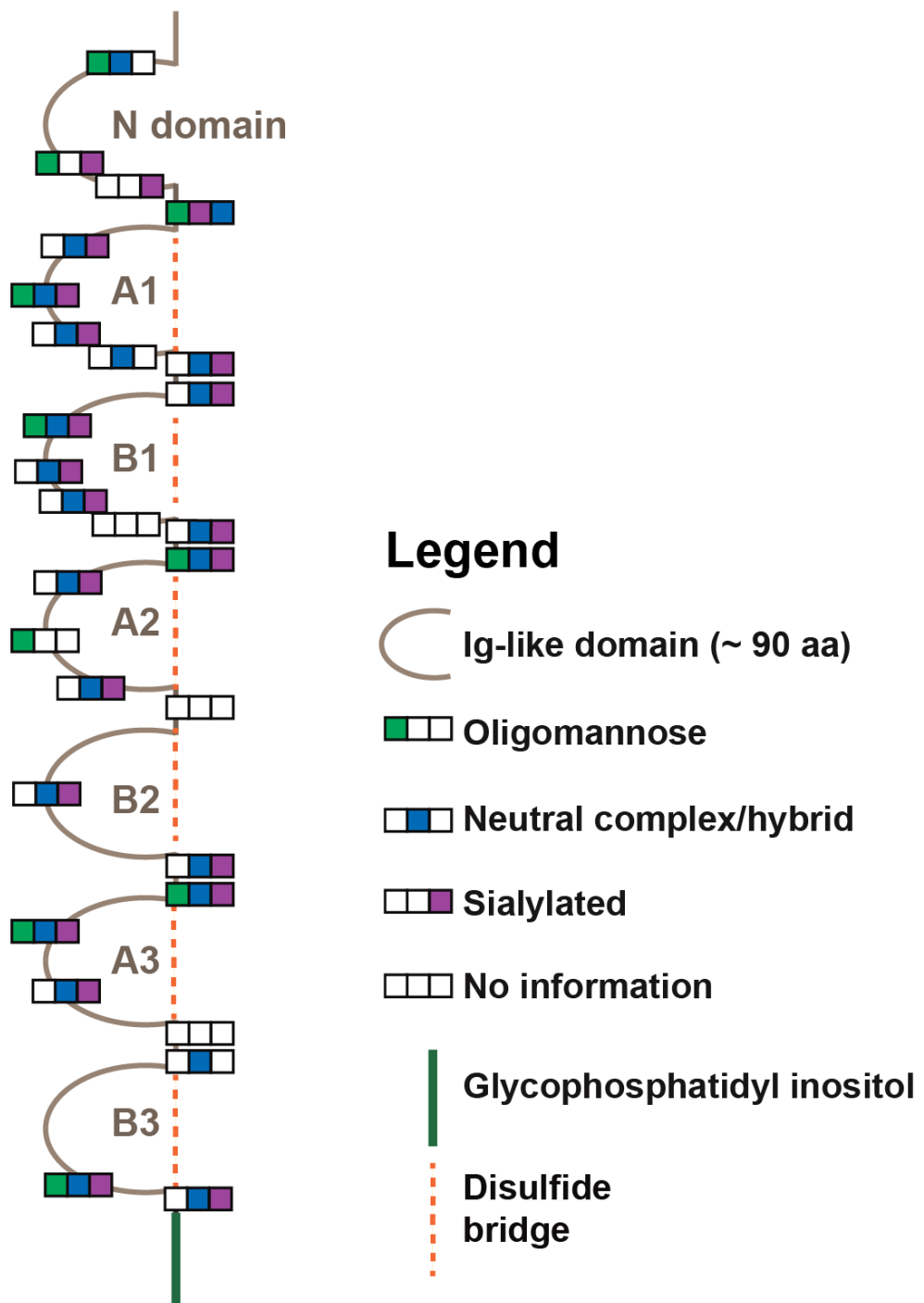
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N-glycosylation of CEA derived from colon adenocarcinoma

Legend

- Ig-like domain (~ 90 aa)
- Oligomannose
- Neutral complex/hybrid
- Sialylated
- No information
- Glycophosphatidyl inositol
- Disulfide bridge

Carcinoembryonic antigen (CEA) is a widely used tumour marker for a variety of different cancers. With 28 potential *N*-glycosylation sites distributed over its seven Ig-like domains CEA is also a highly glycosylated protein. However, to date information on CEA specific glycosylation in health and disease is scarce. To close this gap and evaluate the diagnostic/prognostic potential embedded in CEA glycosylation we used highly sensitive and selective glycomics & glycoproteomics technologies to uncover CEA glycosylation signatures.

Purified CEA was obtained from human colon adenocarcinoma (cell line and tissue), liver metastasis of colon adenocarcinoma and ascites fluid. Glycopeptides obtained by pronase digestion were analysed in a dual LC-setup using reversed phase and porous-graphitized-carbon (PGC) nano-LC-ESI-MS/MS within a single run, while independent PGC nano-LC-ESI-MS/MS glycomics provided a hitherto unprecedented in-depth view on CEA glycosylation.

Glycoproteomics identified 28 *N*-glycosylation sites exhibiting varying occupancy rates of oligomannose, neutral and sialylated *N*-glycans (site-specific glycosylation). These occupancy rates also differed between CEAs from different origins. In all CEAs, however, site Asn309 was found to be not glycosylated. Unexpectedly, in the functionally important N-terminal domain Asn76

was identified to be glycosylated within a non-canonical sequence motif NRQ. CEA has been found to be one of most diversely *N*-glycosylated proteins described to date carrying over 250 different *N*-glycan structures. We identified distinct source dependent glycosylation features in *N*-glycan branching, degree of sialylation and level of bisecting *N*-glycans, indicating that CEA glycosylation provides excellent opportunities to improve currently existing CEA based cancer diagnostics. CEA was also found carrying the sialyl-Lewis X epitope, which is an important Lewis blood group antigen reportedly involved in cell-cell recognition and metastasis. A better understanding of CEA glycosylation signatures will also open novel avenues to study CEA's involvement in cancer progression and metastasis.

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Taming the beast: Standardising porous graphitised carbon based LC-MS glycomics

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Our universal glycomics approach characterises and quantifies glycan structures, from any source, without labels or derivatisation. To separate these glycan mixtures, we use porous graphitised carbon (PGC) as a chromatographic stationary phase, to resolve structurally similar glycan isomers. Previous inter-lab studies have identified that glycomics can benefit from a reduction in technical variability in glycan LC-MS analysis, extending the value of glycomics for probing biology[1].

Following separation, detected glycan structures have associated retention times, however these values are specific to LC conditions and can vary particularly when using PGC due to the column's capacity for redox reactions[2]. As a result, although order of elution is reproducible, actual retention time values are not widely comparable. This challenge has been addressed in fields such as proteomics through the addition of internal retention time standards but standards are not widely available in glycomics due to the structural complexity of carbohydrate chemistry.

A dextran oligosaccharide ladder, similar to that used in reversed phase chromatography of fluorescently labelled glycans, was produced by acid hydrolysis and was characterised by LC-PGC-MS/MS. Eight subunits from 3-10 glucose units (GU) were separated and an equation was fitted to the elution pattern (R^2 >0.999). Analysing *N*-glycans released from eight purified glycoproteins resulted in 212 unique glycan structures being characterised and assigned GU values. These values were found to be more specific for each structure than their retention time value and successfully discriminated closely eluting isomers.

To assess the technical variation of glycan analysis using PGC, a pre-defined mixture of released glycans was analysed including a simulated reduction in retention. Following alignment with the dextran ladder, retention time variation was significantly reduced. The ladder was also used to normalise instrument variation for glycan quantitation, resulting in an average peak area variation (CV) reduction of 30%.

1. Wada Y, Azadi P, Costello CE, et al. Comparison of the methods for profiling glycoprotein glycans—HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study. Glycobiology. 2007;17(4):411-422.
2. Bapiro TE, Richards FM, Jodrell DI. Understanding the Complexity of Porous Graphitic Carbon (PGC) Chromatography: Modulation of Mobile-Stationary Phase Interactions Overcomes Loss of Retention and Reduces Variability. Anal Chem. 2016;88(12):6190-6194. doi:10.1021/acs.analchem.6b01167

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Selenomonas sputigena flagellin glycoproteomics reveals previously not described *O*-glycans and rhamnose fragment rearrangement occurring on the glycopeptides

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The Gram-negative, flagellated, anaerobic, crescent-shaped bacterium *Selenomonas sputigena* is a potential human periodontal pathogen. Information on its virulence factors and underlying pathogenicity mechanisms is scarce. Here we show for the first time that *S. sputigena* produces a diversely and heavily *O*-glycosylated flagellin C9LY14 as a major cellular protein, which carries various hitherto undescribed rhamnose- and *N*-acetylglucosamine linked *O*-glycans in the range from mono- to hexasaccharides. A comprehensive glycomic and glycoproteomic assessment revealed extensive glycan macro- and microheterogeneity identified from 22 unique glycopeptide species. From the multiple sites of glycosylation, five were unambiguously identified on the 437-amino acid C9LY14 protein (Thr149, Ser182, Thr199, Thr259, and Ser334), the only flagellin protein identified. The *O*-glycans additionally showed modifications by methylation and putative acetylation. Some *O*-glycans also carried hitherto undescribed residues/modifications as determined by their respective *m/z* values, reflecting the high diversity of native *S. sputigena* flagellin. We also found that monosaccharide rearrangement occurred during collision-induced dissociation (CID) of protonated glycopeptide ions. This effect resulted in pseudo Y1-glycopeptide fragment ions that indicated the presence of additional glycosylation sites on a single glycopeptide. CID oxonium ions and electron transfer dissociation, however, confirmed that just a single site was glycosylated, demonstrating for the first time that glycan to peptide rearrangement can occur on glycopeptides and that this effect is influenced by the molecular nature of the glycan moiety. This effect was most pronounced with disaccharides. This study is the first report on *O*-linked flagellin glycosylation in *S. sputigena*, revealing that C9LY14 is one of the most heavily glycosylated flagellins described to date. The data have been deposited to the ProteomeXchange with identifier PXD005859

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Determining the role of *Campylobacter jejuni N*-glycosylation in protein stability by *N*-terminomics

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Campylobacter jejuni is the leading cause of gastroenteritis in the developed world, with human infections predominantly acquired via under-cooked or poorly prepared poultry, a host in which the organism is considered a commensal. *C. jejuni* encodes a protein *N*-glycosylation system (Pgl), which targets >100 membrane-associated proteins and is required for both human infection and chicken colonization. Despite this, the exact biological role of this post-translational modification system remains poorly understood. We employed iTRAQ-based labelling to show that loss of the PglB oligosaccharyltransferase (Δ*pglB*), or biosynthetic enzymes that generate the glycan (Δ*pglDEF*), have a limited effect on whole protein abundance relative to wild-type. Changes were largely associated with known glycoproteins, with ~17% of quantified *N*-glycoproteins displaying altered protein abundances. *N*-terminal amine isotopic labelling of substrates (*N*-TAILS) comparisons between wild-type and Δ*pgl* mutants provided evidence for proteolytic processing in close proximity to known sites of *N*-linked glycosylation that was only identified in *pgl* mutant strains. We next performed intact glycopeptide analysis of *N*-glycopeptide enriched membrane protein fractions from wild-type *C. jejuni*, using protein level dimethylation to differentiate those *N*-glycopeptides localised at protein N-termini. Using this approach, we were able to identify 78 unique *C. jejuni N*-glycopeptides, including 2 novel sites. We also provide evidence that the proteolytic activity associated with sites of *N*-glycosylation is limited in the wild-type, further supporting the conclusion that *N*-glycosylation serves to protect *N*-glycoproteins from proteolytic degradation within the *C. jejuni* membrane.

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Automating the release of *N*- and *O*- glycans from glycoproteins

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Glycosylation affects structure, folding, and function of numerous proteins. Aberrant glycosylation has been shown to be associated with different diseases. Currently, there are several approaches for high throughput *N*- and *O*- linked glycan analysis of derivatised glycans such as ultra-performance liquid chromatography (UPLC), liquid chromatography mass spectrometry (LC-MS), capillary gel electrophoresis (CGE) [1]. In our laboratory the routine method that has been found to be the best for glycan isomer separation and structural analysis is porous graphitised carbon liquid chromatography/mass spectrometry (PGC-LC-MS/MS) of reduced *N*- and *O*- glycans released form purified and complex mixtures of glycoproteins[2]. However the sample preparation is still carried out manually. Therefore there is a need for sensitive and robust high-throughput methods for the label-free release of glycans and their preparation for LC/MS analysis.

In our label-free method *N*-linked glycans are released using peptide *N*-glycosidase F (PNGaseF) and *O*-linked glycans subsequently released by reductive alkaline beta-elimination. The standard manual sample preparation for this glycan analysis is very labour-intensive, involving three days of bench work, and also the efficiency of glycan recovery could affect the reproducibility of the method during this workflow. To standardise and automate this process, we have successfully developed a high-throughput sequential *N*- and *O*-glycan release process using the liquid handling robot, Hamilton Nimbus (Bio-Strategy, Australia).

The Nimbus workflow accommodates a use of MPE[2] (positive pressure module) device suited for high-throughput needs (96-well SPE plate) and Hamilton Heater Shaker (HHS). The results demonstrate that highly similar glycan profiles are measured when the Nimbus workflow is compared with the routine manual. The results were based on applying the workflow to analyse the *N*-linked glycans of a highly glycosylated mixture of immunoglobulins.

The development of this automated process for *N*- and *O*-glycan sample preparation from glycoproteins will enable reproducible, high throughput, sensitive analysis to be performed.

1. Huffman, J. E., Pučić-Baković, M., Klarić, L., et al. (2014) Comparative performance of four methods for high-throughput glycosylation analysis of immunoglobulin G in genetic and epidemiological research, Mol. Cell Proteom., 13, 1598-1610.
2. Jensen P. H., Karlsson , N.G., Kolarich D. and Nicolle H. Packer (2012) Structural analysis of N- and O-glycans released from glycoprpoteins. Nature Protocols, 7, 1299-1310.

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Inflammation induces Polysialic Acid in the Brain

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Polysialic Acid is a α2-8-linked sialic acid chain present on cell surfaces in embryonic brains. In adult brains, polysialic acid is only observed in restricted areas where neural plasticity remodeling of neural connections or neural generation is required. Changes in polysialylation pattern are reported to be associated with immune defense and inflammation in the CNS. Opioids such as Morphine-3-Glucuronide (M3G) (a metabolite of morphine) activates neuroinflammation in a manner parallel to Lipopolysaccharide (LPS), compromising opioid-induced analgesia, and the hypothesis of this study is that M3G may affect the polysialic acid expression in neurons. The effects of M3G on the expression of polysialic acid in neurons were studied using

immunocytochemistry and HPLC analysis. It was found that polysialic acid expression was significantly increased following M3G stimulation in neurons similar to LPS when compared to control cells. Polysialic acid was extracted from stimulated cell proteins by endo-neuraminidase digestion and was quantitated using DMB-labeling followed by HPLC analysis. The increased expression of Polysialic acid in M3G-stimulated neurons was validated by western blot analysis. Morphine is known to create neuroinflammation in the CNS. In adults, re-expression of Polysialic acid can be interpreted as an attempt to achieve regeneration. Therefore, upregulation of Polysialic acid in neurons following M3G stimulation may indicate cell recovery from toxic insult

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Enrichment of lowly-hydrophilic truncated *N*-glycopeptides using IP-HILIC-SPE

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Unbiased enrichment of intact glycopeptides from complex peptide mixtures is important to facilitate deep and quantitative glycoprofiling in bottom-up glycoproteomics. Ion-pairing zwitter-ionic hydrophilic interaction liquid chromatography (IP-ZIC-HILIC) SPE provides efficient bulk enrichment of *N*-glycopeptides due to their common highly polar conjugated *N*-glycans[1]. With the discovery of lowly-hydrophilic *N*-glycans in the mammalian glycoproteome spanning the highly truncated paucimannosidic ($Man_{1-3}GlcNAc_2Fuc_{0-1}$)[2] and chitobiose core ($GlcNAc_{1-2}Fuc_{0-1}$)[3,4] type structures, it becomes important to investigate if the corresponding glycopeptides are discriminated against in IP-ZIC-HILIC SPE. We investigated this aspect by first generating relative simple tryptic and non-tryptic mixtures of human glycopeptides carrying a spectrum of glycoforms including chitobiose, paucimannosidic and complex *N*-glycans. The glycopeptides were profiled before and after enrichment using various stationary/mobile phase conditions and column capacities. The resulting LC-MS/MS data was investigated for qualitative and quantitative bias. Tryptic and non-tryptic paucimannosidic peptides including the short $Man_1GlcNAc_2$-peptides appeared to be quantitatively retained whereas chitobiose core type-peptides, in particular the $GlcNAc_1$-peptides were often under-represented in the retentate. IP-ZIC-HILIC-retained tryptic glycopeptides in highly complex mixtures were then investigated relative to matching non-enriched experiments using Byonic-based identification, which supported that tryptic paucimannosidic peptides are often fully retained regardless of the peptide carrier. Finally, *in silico* calculations of the relative hydrophilicity ($\Delta G_{octanol:water}$) of confidently identified chitobiose core and paucimannosidic intact glycopeptides were performed using published glycoproteomics datasets. These multiple approaches provide valuable insight into the retention ability of IP-ZIC-HILIC SPE for truncated lowly-hydrophilic *N*-glycopeptides. We conclude that tryptic paucimanosidic peptides appear to be well-retained under optimised IP-ZIC-HILIC SPE conditions whereas quantitative chitobiose-peptide retention is peptide carrier dependent and thus risk being under-represented in IP-ZIC-HILIC SPE-based glycoproteomics.

[1] Mysling et al. *Anal Chem*. 2010. 82(13):5598.

[2] Loke et al. *Mol Cell Proteomics*. 2017. 16(8):1507.

[3] Trinidad et al. *Mol Cell Proteomics*. 2013. 12(12):3474.

[4] Loke et al. *Biomolecules*. 2015. 5(3):1832.

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Probing synaptic *N*-glycome fluctuations in circadian rhythm

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Circadian rhythm, the "body clock", plays central roles in diverse facets of physiology and is the driver of the sleep/wake cycle. Sleep has been shown to be crucial for memory consolidation, which is supported by the fact that structural and functional changes of neuronal synapses occur during sleep. Thus, we hypothesized that circadian rhythm regulates the synaptic *N*-glycan signatures and that alterations in the synaptic *N*-glycome play functional roles in the synapse modulation that take place during sleep.

With this hypothesis, the *N*-glycome and the underpinning *N*-glycosylation enzymes of neuronal synaptosomes comprising of both pre- and post-synaptic nerve endings of mice brains isolated during the light phase (10 AM, n=5) and during the dark phase (9 PM, n=4) were investigated using deep quantitative glycomics and proteomics. Synaptosomes were isolated by density centrifugation. Synaptosomal enrichment was verified using specific synaptic protein markers. Released *N*-glycans and tryptic peptides from synaptosome membrane protein fractions were analysed by LC-MS/MS. The *N*-glycomics provided a detailed synaptosome *N*-glycan map encompassing 56 biosynthetically-related, mostly asialylated structures displaying prominent core fucosylation and GlcNAc-, mannose-capped and Lewis-type epitopes. Surprisingly few qualitative and quantitative AM-to-PM *N*-glycome differences were observed. Label-free proteomics confidently identified and quantified 193 glyco-enzymes in the synaptosomes of which 42 glycosyltransferases and glycosidases are involved in *N*-glycoprotein biosynthesis. Importantly, circadian regulation of known clock proteins (e.g. BMAL1 and PER1) was observed, which verified the experimental design and the proteomics data. Half (22) of the *N*-glyco-enzymes were significantly regulated during sleep, indicating that these are not rate-limiting in the biosynthetic machinery for *N*-glycoproteins in order to maintain a relatively constant *N*-glycosylation over the sleep-wake cycle. Conclusively, the synaptosomal *N*-glycome does not fluctuate significantly with circadian rhythm, but this study provides a detailed map of synaptic glycan structures for further glycobiological interrogation.

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Enhancement of effects of salmon nasal cartilage proteoglycan on biological function by its modification.

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Proteoglycan (PG) is heavily glycosylated protein, localizes to cell surface and extracellular matrix and has various functions. Recently, it has been gradually revealed that PG interacts with various growth factors and morphogens and regulates cellular functions. In the previous study, we found that salmon nasal cartilage PG (Salmon-PG) increases NHDF proliferation via Erk1/2 activation. Furthermore, to enhance the effect of Salmon-PG on biological function, we created the various modified Salmon-PG and investigated the effects on normal human dermal fibroblast (NHDF). Exp.1: To compare the effect of native and modified Salmon-PG on biological function, we investigated the effect of modified Salmon-PG on the proliferation of NHDF. Modified Salmon-PG does-dependently increased NHDF proliferation and its effect was higher than those of native Salmon-PG. Exp.2: Since we found that Salmon-PG increases NHDF proliferation via Erk1/2 activation in the previous study, we examined the effect of modified Salmon-PG on Erk1/2 phosphorylation by western blot analysis. The modified Salmon-PG (50, 100, 200, 400, 800 and 1600μg/ml) significantly increased Erk1/2 phosphorylation of NHDF compared with native salmon-PG. Exp.3: We analyzed whether the effect of modified Salmon-PG on the cell proliferation of NHDF is mediated through the Erk1/2 signal pathway.   MEK inhibitor suppressed the enhancement of NHDF proliferation by modified Salmon-PG. The overall findings indicate that modified Salmon-PG plays a role as a stronger growth factor in NHDF via Erk1/2 activation than native Salmon-PG, suggesting that modified Salmon-PG strongly contributes to the maintenance of skin homeostasis.

1. Biosci Biotechnol Biochem. 2017 Jul;81(7):1379-1385. Salmon nasal cartilage proteoglycan enhances growth of normal human dermal fibroblast through Erk1/2 phosphorylation. Sano M, Shang Y, Nakane A, Saito T.

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Site specific conjugation of europium chelate to the glycans of monoclonal antibodies: Application for highly sensitive time-gated luminescence detection of Prostate Cancer Cells

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Publish consent withheld

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Mapping protein paucimannosylation across human cancers

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Protein paucimannosylation, a type of truncated *N*-glycosylation with the simple composition $Man(M)_{1-3}GlcNAc_2Fuc(F)_{0-1}$, was previously considered an invertebrate- and plant-specific glycoepitope absent in mammals. However, we recently established that protein paucimannosylation is a significant feature of the human innate immune system[1]. We have also made scattered encounters of paucimannosidic glycans in cancer cells and tissues, but it remains to be systematically investigated if paucimannosylation is a significant cancer glycoepitope. This glycomics-centric study investigates the association of protein paucimannosylation with a wide range of human cancer types and subtypes i.e. brain, breast, liver, colorectal, prostate, ovarian and acute promyelocytic leukemia and matching non-cancerous cultured cells and tissues by mapping the distribution of paucimannosidic *N*-glycans using porous graphitised carbon liquid chromatography tandem mass spectrometry. This *N*-glycomics strategy was able to accurately determine the total level of paucimannosidic glycans within the *N*-glycome and map the relative abundance of the individual paucimannosidic species including M2, M2F, M3 and M3F. The total level of paucimannosidic *N*-glycans varied dramatically across the cancer types (4-35%, n = 3 for all cancers), even within cancer sub-

types as shown for the variation across six breast cancer subtypes (4-13%), but were in general higher than the paucimannosidic levels in non-cancerous cells (0-3%). The core fucosylated M2F and M3F were the predominant paucimannosidic *N*-glycans identified across the studied cancers and clear subcellular-specific differences were observed by the different levels of paucimannosylation in various protein extracts from the same cells. Based on these preliminary correlation-type observations, we conclude that protein paucimannosylation represents significant, but non-uniform glycoepitopes of human cancers. These findings advance our understanding of the glyco-features expressed by human cancers and promote further cause-effect type studies of paucimannosylation in tumorigenesis and metastasis.

[1]Thaysen-Andersen, Venkatakrishnan, Loke, Laurini, Diestel, Parker, Packer. *J Biol Chem* 2015, 290(14), 8789.

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MALDI mass spectrometry imaging of multicellular tumour spheroids: an improved platform for testing novel anti cancer compounds

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Ovarian cancer is the most deadly gynaecological malignancy and is characterised by initial response to first line therapy followed by relapse in more than 50% of cases [1]. Unlike a number of other cancers, survival rates for ovarian cancer have not improved significantly over the last 25 years [2] and the first line treatment strategy for ovarian cancer has not changed significantly over this time period. One of the largest barriers to the development of new treatments is that early testing platforms do not accurately replicate the *in vivo* situation resulting in promising compounds failing at animal or human trials. Multicellular tumour spheroids (MCTS) are an *in vitro* biological model which closely replicates many aspects of the solid tumour and are predicted to bridge the gap between *in vitro* and *in vivo* drug testing [3]. We have established this model in our lab using the ovarian cancer cell line: OV90, in order to test the efficacy of a small molecule inhibitor of CDK4/6. This compound inhibits cancer progression by interrupting the G1-S phase transition in the cell cycle and has exhibited activity against ovarian cancer in monolayer settings. In addition to cell death measurements, we have developed a MALDI mass spectrometry imaging (MSI) workflow for analysis of MCTS. This platform can deliver spatially resolved information about drug penetration, accumulation and metabolism in this biologically relevant model structure providing a window into the solid tumour and how it responds to treatment. The application of MALDI MSI analysis to the MCTS model holds great potential as a drug testing platform and we aim to use this model to test other novel anti cancer compounds into the future. Additionally, establishing MCTS from primary samples can further improve the biological relevance of the model and holds potential for personalised evaluation of drug efficacy.

1. [1] Armstrong, D. K., Relapsed ovarian cancer: challenges and management strategies for a chronic disease. Oncologist 2002, 7 Suppl 5, 20-28.
2. [2] Howlader N NA, K. M., Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z,Mariotto A, Lewis DR, Chen HS, Feuer EJ. , SEER Cancer Statistics Review, 1975-2012. 2015.
3. [3] Kim, J. B., Three-dimensional tissue culture models in cancer biology. Semin Cancer Biol 2005, 15, 365-377.

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Quantitative proteomics of cysteine redox post-translational modifications in myocardial ischemia / reperfusion (I/R) using parallel reaction monitoring mass spectrometry

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Ischemic heart disease involves the occlusion of blood vessels resulting in a cessation of oxygenated blood flow to the heart. This hypoxia, and the necessary reperfusion to salvage surviving myocytes, induce various biochemical changes that cause cellular damage. Notably this includes mitochondrial dysfunction that increases the production of reactive oxygen and reactive nitrogen species (ROS/RNS). This increase in ROS/RNS overwhelms cellular antioxidant defence mechanisms and can alter protein structure / function via various post translational modifications (PTMs). The most common protein PTM induced by ROS/RNS occurs to cysteine (Cys) residues. The broad range of PTM that can occur on Cys can be broken down into two groups, those that are considered enzymatically or chemically reversible and those that are considered 'irreversible'. Reversible modifications include *S*-nitrosylation, *S*-glutathionylation, *S*-acylation, sulfenic acid (Cys-SOH) and intra- and inter-molecular disulfide bonds that influence protein structure, induce redox signalling, act as molecular 'switches' and/or protect Cys residues from subsequent irreversible modification. Irreversible Cys redox PTMs (sulfinic and sulfonic acid; Cys-SO_2H/SO_3H) however are associated with protein dysfunction and/or degradation. A mass spectrometry (MS) technique based on parallel reaction monitoring (PRM) was employed to detect changes in Cys redox PTM in a Langendorff model of myocardial ischemia reperfusion injury (I/R). Due to the low abundance of Cys, and Cys PTM, methods to enrich reversible and irreversible Cys PTM were employed to better profile the changes that occur in I/R. Several Cys sites from a range of proteins underwent a dynamic response in reversible redox PTM during I/R, and irreversible oxidation of some of these Cys sites was also affected. Additionally, the presence of an aminothiol antioxidant (N-mercaptoproionylglycine [MPG]) during reperfusion attenuated I/R injury and irreversible redox modifications. PRM-MS is an effective approach to measuring specific redox modification site abundances during I/R.

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Comprehensive Identification of Crosslinked Peptides using a Multi-crosslinker, Fragmentation and Data analysis Approach

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Crosslinking mass spectrometry (XLMS) is a method to study protein-protein interactions. It combines chemical crosslinkers, protease digestion and tandem mass spectrometry, whereby fragmentation of the crosslinked peptides is used to discover intra- and inter-protein crosslinks. Early XLMS studies used non-MS cleavable crosslinkers such as BS^3, BS^2G and DTTSP. However, new MS-cleavable linkers, such as DSSO, DSBU and Protein Interaction Reporter (PIR), have helped address many of the prior limitations of XLMS, such as co-fragmentation of crosslinked peptides. They allow predictable generation of high-intensity reporter ions which new generation crosslink identification programs such as MeroX, XlinkX and ReAct can use to increase speed, and confidence of identifications. Here, we have used a multi-crosslinker, fragmentation and program approach to address two major aims. Firstly, we investigated the enzyme/substrate interaction between Npl3p and its methyltransferase Hmt1p. Secondly, we used this combined analysis approach to understand how different crosslinkers (DSBU/DSSO), fragmentations (CID+ETD/SteppedHCD), programs (MeroX/XlinkX 2.0), and algorithms (Precursor and Reporter-Ion) impacted the results. From this study we have defined that the interaction between Npl3p and Hmt1p involves the intrinsically disordered "SRGG" region of Npl3p and the N-, C- termini and S-adenosyl methionine binding site of Hmt1p. For the first time, we have also shown direct evidence for Npl3p dimerization occurring at the "SRGG" region. We have also confirmed higher order multimerisation of Hmt1p in accordance to the known structure. Importantly, we demonstrate from our multi-crosslinker, fragmentation and program comparisons that one-type of analyses may not capture all identifiable crosslinks. This is attributed to a combination of biases inherent to either a MeroX/DSBU approach or an XlinkX/DSSO approach.

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Mass spectrometry based interrogation of the IMiD dependent zinc-finger degron landscape

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First administered in the late 1950s as a mild sedative, thalidomide led to the birth of thousands of children with multiple birth defects. Despite their teratogenicity, thalidomide and closely related immunomodulatory drugs (IMiDs) are now a mainstay of cancer treatment. Thalidomide binds to the Cullin RING E3 ubiquitin ligase CUL4-RBX1-DDB1-CRBN ($CRL4^{CRBN}$) and promotes ubiquitination and degradation of key therapeutic targets such as IKZF1/3 and Ck1 in a molecular glue like mechanism. $CRL4^{CRBN}$ was further shown to exert ubiquitin dependent or independent functions on GLUL, CD147-MCT1, MEIS2, and ZFP91, however, none of these targets can account, in full, for the desired therapeutic and undesired adverse effects of IMiDs.

The human genome encodes for over 700 C_2H_2 zinc-finger (ZnF) proteins, which share high homology with the known targets IKZF1, IKZF3, and ZFP91, and contain thousands of potential thalidomide-CRBN binding ZnF degrons. Many of these ZnF proteins are expressed at low levels or in a lineage specific manner, and are therefore hard to detect by most methods. To interrogate the potential target repertoire of IMiDs, we set out to develop novel mass spectrometry-based proteomics approaches and screen a panel of human cell lines covering multiple lineages.

We will present 1) novel mass spectrometry-based proteomics approaches that allow profiling of 7 individual compounds inducing protein degradation – such as IMiDs – in a single mass spectrometry experiment at a depth of > 10,000 proteins and > 160,000 unique peptides. 2) data on the currently unexplored substrate repertoire of IMiDs across lineages, which includes dozens of novel ZnF proteins being targeted by these drugs, several of which have been linked to human disease. 3) newly identified IMiD-dependent ZnF substrates that share amino acid residues that are critical for the interaction with $CRL4^{CRBN}$, and IMiD-dependent degradation and will guide future development of novel ZnF targeting analogs.

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Proteomics investigations reveal molecular similarities and differences between human and rat retinas under glaucoma conditions.

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Glaucoma is a progressive neurodegenerative disease of the eye that represents one of the major causes of irreversible blindness worldwide. This complex degenerative disorder is characterised by progressive loss of retinal ganglion cells (RGCs) in the inner retina. While several risk factors have been identified that are associated with premature loss of RGCs, high intraocular pressure is currently the most significant risk factor in glaucoma. The molecular mechanism(s) that result in RGC dysfunction in various optic neuropathies however remain ill-defined. Greater understanding of the underlying neurodegenerative processes is crucial for the development of effective therapeutic strategies for glaucoma.

In this study, we sought to investigate the molecular basis of glaucoma pathogenesis by taking a systems-level perspective of the human retinal proteome and compare it with experimental glaucoma animal model using unbiased quantitative proteomics approaches. Multiplexed Tandem Mass Tag based proteomics (TMT-MS3) was carried out on retinal and vitreous humour tissues collected from glaucoma patients and age-matched controls (n: 20). Rat model of glaucoma was generated in the lab using repetitive microbead injections into the anterior chamber of the eye to help increase the intraocular pressure and retinal tissues analysed using TMT proteomics analysis (n: 10). This was followed by comprehensive functional pathway and protein network interaction analysis.

About 5000 proteins were quantified from both the human and experimental glaucoma model. Pathway analyses of differentially regulated proteins indicated specific activation of classical complement pathway and cholesterol metabolism in human glaucoma retinas suggesting an innate inflammatory response. Molecular dysregulation of oxidative phosphorylation, protein misfolding and glutathione biosynthesis pathways were identified in both the human and animal glaucoma model.

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An investigation into MALDI imaging sample preparation compared to DESI imaging for multimodal MSI in Pre-clinical Breast Cancer Research

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Breast cancer is a complex and heterogeneous disease that has distinct biological features and clinical characteristics. The understanding of breast cancer, which is the most frequently diagnosed form of cancer and the second leading cause of death in Western women, has greatly profited from research using genetically modified mouse models[1].

Mass spectrometry imaging (MSI) is an established analytical tool for biomolecular research which can accurately determine the spatial location of molecules in a tissue section.

In this study, we present data comparing several sample preparation protocols for MALDI MSI and contrasting with DESI MSI using mouse breast normal and tumour samples.

Experiments were conducted on normal and tumour samples from the polyoma middle T oncoprotein (PyMT) mouse model of breast cancer, which were sectioned using a cryotome and deposited onto standard microscope slides preserved at -80C degrees until analysis by mass spectrometry.

The tissue sections were analyzed firstly by MALDI MSI using a SYNAPT G2-S*i* mass spectrometer with a MALDI source operating with a solid-state diode-pumped ND:YAG laser using a repetition rate of 1 KHz. Consecutive tissues were then analyzed by DESI MSI, using a modified Prosolia source, directly mounted onto the SYNAPT G2-S*i*.

With DESI control tissue datasets, the highest signals were generated by the triglyceride molecules directly from the tissue sections. However, molecular profiles changed considerably in the breast tumor samples, with an increase in intensity of signal for the detection of phosphatidylcholine . This was not observed with the datasets generated by MALDI MSI. Indeed, with MALDI triglyceride molecules were clearly shown in both normal or cancerous tissue under the sample preparation conditions used. The observed difference in lipid profiles using MALDI between the tissue types were more subtle and were found to be related to differences in phospholipids.

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Temporal quantitative profiling of ΦX174 and host-cell proteomes during lytic infection

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The potential for bacteriophage to be used for industrial and medical purposes are on the cusp of breaking onto the market and to be able to model, control, engineer, and use viruses in medical and industrial purposes we must be able to measure their phenotypic responses over time. Unfortunately, there is currently limited understanding regarding host-cell proteomic response to lytic bacteriophage infection. This study explores lytic bacteriophage infection using the simple 11-gene model bacteriophage ΦX174 and its host *Escherichia coli* (strain C). A 10-plex TMT quantitative LC-MS strategy was used to probe the proteomic response to the ΦX174 infection across a five-point time-course covering the entirety of the 75-minute lytic infection.

2185 non-redundant proteins were identified at 1% false-discovery rate (FDR), 1764 of which were quantifiable across all samples. Using two-sample t-tests with multiple testing corrections, 706 proteins were observed to be differentially regulated (q-value < 0.05) across grouped time-points (early-stage, mid-stage, and late-stage) and/or phage infected and control samples. All 11 ΦX174 proteins were quantifiably detected, including the previously elusive phage lysis protein E. This enabled temporal mapping of ΦX174 proteome abundance during infection. Interestingly, we observed two host *E. coli* heat shock proteins, IbpA and IbpB, that are the dominant response to bacteriophage expression and have not previously been reported in this context. We are now undertaking functional categorization to reveal the host-cell response to infection.

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Backwards and forwards in time: Urinary proteomics of anterior cruciate ligament injury

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Anterior cruciate ligament (ACL) and associated knee injuries severely impact on an individual's opportunity to resume previous physical activity levels and may also increase the risk of developing knee osteoarthritis. While magnetic resonance imaging provides excellent diagnostic information on the specific structures involved in the injury, it is expensive and therefore not practical for providing repeated follow-up information after ACL reconstruction. Recent advances in protein profiling approaches including liquid chromatography–tandem mass spectrometry (LC–MS/MS) provides evidence to suggest that novel diagnostic biomarkers of knee injury and recovery may be detectable. Hence, this research focuses on interrogating the urinary proteome, using a quantitative data-independent acquisition mass spectrometry based proteomics technique (SWATH™), to reliably quantify urinary proteins associated with acute ACL injury and repair status post-surgery. The MS data obtained from pre- and post-operative urine samples from 14 confirmed ACL injured patients were analysed and resulted in spectral library generation of more than 500 proteins. Multivariate statistical analysis revealed proteins that were associated with the injured cohort and proteins that could be indicative of tissue recovery outcomes. In addition, the potential influence of the pre-operative injury period on the protein abundance was investigated. The results indicated that the pre-operative injury period may affect the levels of a subset of proteins. Significantly overrepresented biological process, cellular component and molecular function gene ontologies related to these candidate proteins were also determined. Such biomarkers may provide additional tools for clinicians in the evaluation of both the initial injury but importantly, tissue recovery following surgery.

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ERK1/2 is a key regulator of early myocardial ischemic damage

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Cardiovascular disease is one of the leading causes of death with ischemic heart disease (IHD) the largest contributor. The long-term outcomes following IHD are directly attributable to the duration of ischemic insult, with short periods of ischemia (<20 mins) producing reversible contractile dysfunction, while prolonged ischemia (>30 mins) results in irreversible contractile injury. In previous studies we utilised large-scale phosphoproteomics to monitor the activation/repression of signalling pathways over an ischemic time course spanning 0-60 minutes. ERK1/2 was identified to be significantly upregulated within the first 2 minutes of ischemia only, suggesting a significant role of ERK1/2 during reversible ischemic damage. To investigate the importance of ERK1/2 during the early phases of ischemia, an upstream MEK inhibitor (U0126) was introduced during the ischemic period, with subsequent reperfusion to monitor the return of contractile function. Hearts were subject to one of the following conditions; 2 minutes of ischemia with/without U0126 (2I, 2IU); 2 minutes of ischemia with/without U0126 followed by 5 minutes of normal reperfusion (2I/5R, 2IU/5R) or; 2IU followed by 5 minutes of reperfusion with U0126 (2IU/5RU). Haemodynamic comparison showed that 2IU/5R had significantly poorer function compared to 2I/5R (54.8% ± 7.3 % RPP vs 93.1% ± 2.8% RPP) while 2IU/5RU did not (86.8% ±9.2%), suggesting that ERK1/2 inhibition during ischemia and reperfusion has no effect on function. If ERK1/2 is inhibited during ischemia but re-activated during reperfusion it causes significant deterioration of contractile function. This suggests activation of a compensatory mechanism/signalling pathway in the absence of ERK1/2 activity. Using a combination of phosphoproteomics and Western Blot, we found that AMPK contributed in a compensatory way to ERK1/2 inhibition. The ability to activate AMPK, appears to circumvent the need for ERK1/2 regulation, however activation of both AMPK and ERK1/2 is detrimental to the heart, leading to poor functional outcomes.

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T-cadherin: A potential biomarker for healthy adipose tissue

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Publish consent withheld

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Displacement chromatography within online 2D-LC-MS is improving identification of proteins from low microgram amounts

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Displacement chromatography, an elution mode already introduced more than 70 years ago, has been described to offer some advantages over gradient chromatography like high separation efficiency, no need for salts in ion exchange chromatography and enrichment of low abundant molecules. Recently our group demonstrated that displacement chromatography is applicable and

beneficial also for bottom up proteomics of complex protein mixtures (Trusch et al. 2012). However, in that online two-dimensional liquid chromatography mass spectrometry (2D-LC-MS) approach the total run time was unacceptable long. In a new study a much smaller cation exchange column (CEC) was chosen with a bed volume of 120 nl and a total binding capacity of approximately 5 µg tryptic peptides. The CEC was integrated into a LC-MS system. The performance of this 2D-LC-MS system for identifying the proteins in a complex tryptic peptide mixture (HeLa protein digest standard) was compared, using either the displacement (DM) or the gradient mode (GM) in the first dimension. With DM a significantly better separation efficiency was achieved, especially for peptides which are doubly positive charged in the eluent at a pH of 2.3 and are the majority of tryptic peptides in mammalian proteomes. The better separation in DM is responsible for a better reproducibility, larger number of identified peptides (> 2.5-fold increase for doubly charged peptides) and proteins including higher protein sequence coverages. In conclusion, the study demonstrates that the displacement mode for CEC in the first dimension of 2D-LC-MS is clearly better than the gradient mode and can already be used for total peptide amounts less than 5 µg.

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SWATH-MS spectral reference library species conversion with the R package 'dialects'

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SWATH-MS is a mass spectrometry (MS) approach that measures all theoretical peptide fragments. The resulting file is extremely complex and identification of peptides generally relies on an independently generated spectral reference library (SRL). The SRL comprises a table of transitions, defined though a series of data-dependent acquisition (DDA)-MS runs. The SWATH-MS data are searched with the SRL for the presence of the predefined peptides and associated fragments. Creation of an SRL is a lengthy process with large computational requirements. Additional searching of DDA-MS files with species other than the original sample is often avoided. This approach can preclude using large well characterised SRLs designed in one species from the use with alternate species. To overcome this, we developed an R package named 'dialects' (data independent acquisition library editing to convert the species) for SRL conversion between species. This package contains five core functions: 1. import a UniProt protein sequence database (fasta file); 2. import a PeakView/OneOmics or OpenSWATH SRL; 3. perform an *in silico* tryptic digestion on UniProt proteins; 4. convert the species of the SRL to that of the digested UniProt database, only for full sequence identity peptides; 5. export the new SRL in either PeakView/OneOmics or OpenSWATH format. This R package aids the creation of comprehensive SRLs, without the need for repeated MS runs and/or reprocessing of MS searches. This reduces the time to convert between species of an SRL and expands the utility of large, well characterised SRLs.

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Proteomics exploration in skeletal muscle following physical trauma

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Muscle injury is a prevalent cause of debilitation for workers, athletes, and the public generally at home and in motor vehicle accidents. These incidents cause suffering to patients. To date, the general processes associated with skeletal muscle injury have been described. In spite of these investigations into skeletal muscle injury, it is still unclear as to the specific mechanisms of injury progression and factors affecting the initiation of the recovery process. It is therefore necessary to conduct research aimed at providing more detailed insights into the fundamental factors and mechanisms that regulate the activity of skeletal muscle cells following injury.

Methodology

The project applied proteomics based approaches in in vivo model. Specifically, a rat impact contusion model was utilized to model impact trauma. Dynamic global protein profiling using, LC-MS/MS was performed at 6h, 12h, 1, 3, 7 and 14 days on tissue homogenates to identify factors that are associated with the initial recovery response following injury. Specifically, pooled protein samples from all of the animals were fractionated by LDS-PAGE and in-gel digestion, while 35 individual samples were fractionated by filter-aided sample preparation. Liquid chromatography tandem mass spectrometry (LC-MS) analysis was performed. Pooled samples were analysed by data-dependent acquisition, whereas each individual sample was analysed by data-independent acquisition. Data explorations were performed using multiple tools.

Results

1. Differentially expressed proteins were identified in each post-injury time points based on statistical analysis and 2 fold change.
2. Protein-protein interaction, and GO enrichment and KEGG pathway of over regulated-proteins at each post-injured time points were determined by direct visual network.

Conclusion

Global proteomics profiling revealed comprehensive insights into the dominant biological processes that occur in skeletal muscle after injury. The key protein-protein interactions and biological networks that underpin muscle regeneration have been defined in my study.

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Proteomics of the uterus of lactating dairy cows and its effect on fertility

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Dairy cows' fertility has been shown to be declining in many countries, possibly due to selection based on their milk production traits, without enough consideration for reproductive performance. Economic repercussions of this impaired fertility warrant investigation into its underlying mechanisms.

Many factors have been proposed to be relevant, including the animals' energy balance. Negative energy balance after calving, to a high extent due to milk production, predisposes cows to nutritional and hormonal imbalance and these imbalances have been associated with decreased fertility.

Our previous work found that in New Zealand Holstein-Friesian dairy cows 30% of the embryo losses occurred before day 7 after insemination. It was also found that only 29.6% of cows inseminated on their first oestrous cycle after calving (OAC) carried a viable embryo at day 7, compared to 60% for those inseminated on their third or more OAC.

The aim of this study is to investigate proteomic and metabolomic differences in the uterine environment between these two physiological states through analysis of uterine flushings at day 7 after insemination.

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MethylQuant: a tool for sensitive validation of enzyme-mediated protein methylation sites from heavy-methyl SILAC data

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The study of post-translational methylation is hampered by the fact that large-scale LC-MS/MS experiments produce high methylpeptide false discovery rates (FDRs).1 The use of heavy-methyl SILAC can drastically reduce these FDRs; however this approach is limited by a lack of heavy-methyl SILAC compatible software. To fill this gap we recently developed MethylQuant. Here we describe an updated version of MethylQuant, which we have recently made available.2 We demonstrate its methylpeptide validation and quantification capabilities and provide guidelines for its best use. Using reference heavy-methyl SILAC datasets, we show that MethylQuant predicts with statistical significance the true or false positive status of methylpeptides in samples of varying complexity, degree of methylpeptide enrichment, and heavy to light mixing ratios. We introduce methylpeptide confidence indicators – MethylQuant Confidence and MethylQuant Score – and demonstrate their strong performace in complex samples characterized by a lack of methylpeptide enrichment. For these challenging datasets, MethylQuant identifies 882 of 1165 true positive methylpeptide spectrum matches (i.e. >75% sensitivity) at high specificity (<2% FDR), and achieves near-perfect specificity at 41% sensitivity. We also demonstrate that MethylQuant produces high accuracy relative quantification data that is tolerant of interference from co-eluting peptide ions. Together MethylQuant's capabilities provide a path toward routine, accurate characterizations of the methylproteome using heavy-methyl SILAC.

1.   Hart-Smith G, Yagoub D, Tay AP, Pickford R, Wilkins MR (2016) Large-scale mass spectrometry-based identifications of enzyme-mediated protein methylation are subject to high false discovery rates. Molecular & Cellular Proteomics, 15: 989-1006.
2.   Tay AP, Geoghegan V, Yagoub D, Wilkins MR, Hart-Smith G (2017) MethylQuant: a tool for sensitive validation of enzyme-mediate protein methylation sites from heavy-methyl SILAC data. Journal of Proteome Research, In press. DOI:10.1021/acs.jproteome.7b00601

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Optimising the number of Variable SWATH Windows

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Variable window acquisition for SWATH analysis has enabled comprehensive analysis of complex matrices while maintaining the acquired mass range without affecting cycle times. Variable SWATH windows allow smaller Q1 windows in m/z dense regions where many peptide precursors are measured, and wider windows where fewer precursors are measured. This results in superior quantitative data and increased peptide identifications. The original SWATH method used 32 fixed windows, with 64 fixed windows now relatively common. More recently, 100 variable windows have been advocated. However, increasing the number of windows comes at a cost to either cycle time or points across a chromatographic peak, both of which have an impact on the quality of data acquired.

To maintain the number of points across a peak, thereby maintaining quantitative reproducibility when the total number of SWATH windows are increased, cycle time would need to be decreased. However, decreasing the cycle time negatively affects the quality of spectra acquired which could lead to a reduction in peptides identified and quantified.

Alternatively, keeping cycle time constant while increasing the number of SWATH windows results in fewer points across a peak that may negatively affect quantitation reproducibility by increasing variability.

For this study, a number of variables were tested including; cycle time, collision energy spread, and points across a peak. The aim was to achieve the maximum numbers of peptides and proteins identified reproducibly across a 90-min LC run, whilst finding the optimal balance between cycle time and points across a peak.

Comparison of Different Correlation Metrics for Protein Correlation Profiling of Yeast Protein Complexes

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Large-scale studies of protein complexes often involve affinity purifications of tagged-proteins 'one-at-time' followed by LC-MS/MS. Recently, large-scale coverage of the complexome could be achieved through protein correlation profiling (PCP), without the need for tagged-proteins. A typical PCP experiment involves the use of size exclusion chromatography to separate protein complexes based on their size. Proteins in the same complex are co-eluted and are likely to have high correlation in protein abundance profiles across all the fractions. The aim of this study is to compare three different correlation metrics for the identification of protein complexes in *Saccharomyces cerevisiae*. These metrics include the Pearson correlation coefficient, Spearman's rank correlation coefficient, and Maximal Information Coefficient (Reshef *et al.* 2011). A high confidence set of protein complexes in *S. cerevisiae* curated by Benschop *et al.* (2010) was used for benchmarking. From the analysis of seven PCP datasets, the Spearman's correlation consistently identified more protein complexes with higher average correlation per complex, outperforming the other two metrics. An application of PCP is to identify changes in protein complexes between mutant and wild type yeast strains, which could be identified through changes in Spearman's correlation pattern between mutant and wild type. Two knockout mutants of lysine protein methyltransferases (*efm4Δ* and *efm7Δ*), which solely targets the methylation of eukaryotic translation elongation factor 1α (eEF1α), affected the correlation profile of proteins in the eEF1α complex. The knockout of arginine protein methyltransferase (*hmt1Δ*), which catalyze the methylation of Npl3p, also led to changes in Npl3p's correlation with known partners. The above suggests protein methylation could affect protein-protein interactions and complex formations. Future directions would involve peaks identification from protein abundance profiles to reduce noise (Scott *et al.* 2014) and the use of machine learning to predict direct protein-protein interactions (Drew *et al.* 2017).

1. Benschop, J. J.; Brabers, N.; van Leenen, D.; Bakker, L. V; van Deutekom, H. W.; van Berkum, N. L.; Apweiler, E.; Lijnzaad, P.; Holstege, F. C.; Kemmeren, P. A consensus of core protein complex compositions for Saccharomyces cerevisiae. Mol. Cell. 2010, 38 (6), 916–928.
2. Drew, K.; Müller, C. L.; Bonneau, R.; Marcotte, E. M. Identifying direct contacts between protein complex subunits from their conditional dependence in proteomics datasets. PLOS Comput. Biol. 2017, 13 (10), e1005625.
3. Reshef, D. N.; Reshef, Y. A.; Finucane, H. K.; Grossman, S. R.; McVean, G.; Turnbaugh, P. J.; Lander, E. S.; Mitzenmacher, M.; Sabeti, P. C. Detecting novel associations in large data sets. Science 2011, 334 (6062), 1518–1524.
4. Scott, N. E.; Brown, L. M.; Kristensen, A. R.; Foster, L. J. Development of a computational framework for the analysis of protein correlation profiling and spatial proteomics experiments. J. Proteomics 2015, 118, 112–129.

Proteomics analysis of specific brain regions from Alzheimer's disease model mice at the early stage of disease pathology

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Alzheimer's disease is considered a progressive multifarious neurodegenerative disorder and known as a foremost cause of dementia in late adult life. The pathogenesis of Alzheimer's disease (AD), especially the early events of AD pathology, remains undetermined, chiefly due to the complexity of AD and failure in diagnoses of the disease in the early stages. Proteomics analysis has provided comprehensive insights to investigate the complex cellular activity in the brain both in human and animal studies, however, only limited studies were performed on the brain of the early AD individuals or young transgenic AD animal models. Here, we report the most comprehensive proteomic analysis of the most vulnerable brain area (hippocampus, frontal and parietal cortex) and less susceptible brain region (cerebellum) of 2.5-month-old of APP/PS1 transgenic mouse model of the AD and age-matched control animals. Multiplexed Tandem Mass Tag based proteomics was carried out on different brain region of APP/PS1 and wild-type animals. Further, comprehensive functional pathway and protein network interaction analysis performed using Ingenuity, STRING, and Panther analysis tools. Selected differentially modulated proteins were validated using western blotting. Approximately 4500 proteins were identified and quantified (1% FDR) from each brain region. Our data revealed that 471, 352, 226 and 33 proteins were up-regulated and 374, 466, 251 and 31 proteins were down-regulated in the hippocampus, frontal cortex, parietal cortex, and cerebellum respectively. In agreement with previous findings in the literature, the cerebellum is the least affected brain region in AD, despite beta-amyloid protein being identified as consistently up-regulated in all 4 different brain regions in APP mice model as compared to the control animals. Further, pathway enrichment analysis of differentially expressed proteins revealed that the most enriched biological pathways in the affected areas were associated with endocannabinoid signalling, glutamatergic synapse, GABAergic synapse, calcium signalling and mitochondrial dysfunction.

Non-protein amino acids and neurological disease: their detection in human proteins and effects

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Neurological diseases are hallmarked by protein aggregation and neuronal apoptosis in major diseases such as; Parkinson's; Alzheimer's and Motor Neurone disease. In 90% of disease cases there is no underlying genetic abnormality that defines pathogenesis. Certain non-protein amino acids (NPAAs) have been presented by the literature to be a possible causative agent in these sporadic cases, such as the NPAA beta-methyl-L-alanine (BMAA) [1] or the therapeutic drug used in Parkinson's disease (Levodopa) [2]. The mechanism underlying these NPAAs is hypothesised to be miss-incorporated into proteins in place of protein amino acids, modifying native-protein structures causing misfolded proteins resulting in neuronal aggregates and apoptosis. This was investigated by the use of neuronal neuroblastoma cells (SH-SY5Y) utilising label free and Tandem Mass Tag (TMT) labelling approaches with the use of high resolution mass spectrometry, both data-dependent and independent approaches combined with; exclusion list generation to delve beyond the dynamic limit of detection. This is novel due to protein hydrolysis and amino acid assays being used to infer incorporation in the analytical fields of toxicology to date. These data generated provides proof of NPAA incorporation by use of novel positive controls and spectral evidence including; spectral library generation. These data also provide workflow for the incorporation of NPAAs in clinical samples.

1. 1:Cox, P. A., D. A. Davis, D. C. Mash, J. S. Metcalf and S. A. Banack (2016). "Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain." Proceedings of the Royal Society B: Biological Sciences 283(1823).
2. 2:Chan, S.W., Dunlop, R.A., Rowe, A., Double, K.L. and Rodgers, K.J., 2012. L-DOPA is incorporated into brain proteins of patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro. Experimental neurology, 238(1), pp.29-37.

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Identification of a kynurenine-modified HIV epitope using immunopeptidomics

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Background

A small fraction of HIV-infected patients known as long-term non-progressors (LTNPs) maintain very low levels of plasma HIV without antiretroviral therapy. The majority of these possess HLA alleles such as HLA-B*57:01 that are thought to offer superior T-cell mediated immunity where intracellular pathogen-derived peptides are presented for scrutiny to CD8+ T cells leading to eradication of infected cells. Here we discuss the identification and detailed characterization of HLA-B*57:01-restricted post-translationally modified (PTM) peptides, specifically focusing on a kynurenine-modified peptide due to its novelty.

Methodology

Cells expressing HIV envelope (env) were transfected with HLA-B*57:01 followed by immunoaffinity purification of HLA class I molecules. HLA-bound peptides were fractionated and analysed using SCIEX 5600+ TripleTOF. HIV peptides were validated by matching the MSMS and RT to synthetic peptides. HLA-B57 in complex with native and PTM peptides were crystallised to study structural properties followed by biochemical assays to study formation of peptidyl-kynurenine.

Results

Here we report on the identification of over 8700 naturally presented class I HLA-B*57:01-bound peptides including seven derived from HIV-env. Both native and PTM forms of two key peptides were identified. Notably, we identified a kynurenine modified HIV peptide and show using biochemical assays that this can be recapitulated enzymatically from native peptide. We study the binding of such HLA-B*57:01-restricted peptides at a structural level and examine their immunogenicity in preliminary functional studies in HLA-B*57:01+ HIV-infected and HIV-naïve humans.

Conclusions

In summary, we have identified several novel HIV-env-derived peptides including native and PTM forms. Our study demonstrates the potential for kynurenine containing epitopes to be recognized by T-cells, leading the way for other researchers to look for responses against such modified epitopes in their HIV cohorts as well as from other conditions where inflammation may drive the incorporation of kynurenine into other viral, tumour or autoantigenic epitopes.

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SP3-DIA on a Q-Exactive HF enables high-throughput and miniaturised screening of cardiac organoids

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The goal of high-throughput quantitative proteomics is to precisely quantify every protein in a sample in the shortest possible time. Here, we compared data-dependent acquisition (DDA) versus data-independent acquisition (DIA) on a Q-Exactive HF with the aim of achieving the greatest proteome depth and quantitative accuracy/precision. We initially generated spectral libraries containing 135,075 peptides representing 10,119 proteins by fractionating digested HeLa lysates. Unfractionated single-shot analysis was subsequently performed in triplicate with either DDA or DIA and analysed with Match Between Runs in MaxQuant or Spectronaut, respectively. DIA quantified significantly more peptides in all three triplicates than DDA (~85,000 vs ~57,000), and quantified more peptides with less than 20% coefficient of variation. At the protein level, DIA quantified slightly more proteins however surprisingly; the reproducibility of DDA was slightly better presumably due to the more advanced label-free quantification (LFQ) features of MaxQuant. We next modified the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) approach using paramagnetic beads with 96-well plate magnets to perform proteomic analysis of 1 mm cardiac organoids. Combined with spectral libraries and DIA, this approach enabled us to quantify >6,000 proteins per 2 h run. We applied this approach to; i) characterise organoid maturation, ii) compare matured organoids to human myocardium and, iii) perform a screen of novel cardiac regeneration compounds in pre-clinical trial.

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Identification of T cell epitopes involved in adverse reactions to β-lactam antibiotics

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Although widely used, penicillins cause a range of adverse drug reactions ranging from cutaneous reactions to anaphylaxis in 1-10% of individuals treated. These reactions are generally classified as idiosyncratic and involve inappropriate activation of the immune system during drug administration. Depending on reaction phenotype, a range of immune cell types have been implicated, including both CD4 and CD8 T cells. In healthy individuals, CD8 and CD4 T cells are normally activated via the presentation of pathogen derived peptides on the surface of antigen presenting cells by MHC class I and II molecules respectively. In contrast, self-derived peptides presented by the MHC are usually ignored. The ability of β-lactam antibiotics, including penicillins, to covalently modify proteins is well established, and the prevailing hypothesis for T cell activation in adverse reactions to penicillins is the presentation of immunogenic penicillin-modified self-peptides by MHC molecules. We have recruited a cohort of penicillin allergic patients from The Alfred Hospital Allergy Clinic (n=10), who have experienced a range of adverse reactions to penicillins including amoxicillin. In preliminary studies we have isolated drug responsive T cells from a patient who experienced an accelerated response to amoxicillin that are activated via the MHC class I molecule HLA-A*02:01. Using a B-lymphoblastoid cell line derived from this patient, we are exploring the peptides presented by HLA-A*02:01 of untreated and drug treated cells via an immunoaffinity purification, LC-MS/MS workflow. Evidence for drug haptenated peptides will be discussed. These analyses will form the foundation of a broader characterisation of potential drug-induced T cell epitopes across the patient cohort.

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Optimizing red blood cell protein extraction for proteomic analysis

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Blood is a rich source of protein biomarkers. However, it is one of the most analytically challenging matrices due to its high compound complexity. This complexity can be reduced by separation of cellular elements [e.g. red blood cells (RBCs), peripheral monocytes] from plasma. Many biomarker assays and studies have been conducted on plasma (or serum), but not on RBCs. Alpha-synuclein (SNCA), a known neuropathological biomarker of Parkinson's disease, is an abundant RBC protein. Therefore, RBCs may provide a valid avenue for proteomics and the study of neurodegenerative diseases. However, proteomics analysis of RBCs still represents an analytical challenge, especially due to the large dynamic range and the high abundance of lipids. To determine the most efficient way to extract the RBC proteome, we evaluated six different trypsin digestion methods, namely (1) Urea, (2) Acetone followed by Urea, (3) Sodium deoxycholate (4) Acetone followed by sodium deoxycholate, (5) Acetonitrile and (6) Acetone followed by acetonitrile. We further established a standard curve of ^{15}N-labelled α-synuclein to quantify endogenous α-synuclein, and also screened the RBC proteome for other neuropathological biomarkers. After RBC protein extraction and the tryptic digest, lysates were analyzed on an Agilent 6495 QQQ instrument in dynamic MRM mode, injecting 3μg and 10μg of total protein. The results show that RBC protein extraction with sodium deoxycholate gives the best proteome coverage with the highest peptide signal intensities when 3μg of total protein is injected. Extraction with acetonitrile is the 2nd best method, partially enriching a different set of peptides. Here, the 10μg injection gave the best results. Besides endogenous alpha-synuclein, protein deglycase DJ-1 (PARK7) and superoxide dismutase (SOD1) were also identified in RBC, all playing pivotal roles in neurodegenerative diseases. These results highlight the potential of RBCs for neuroproteomics.

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Optimising protein extraction for the study of the alpha-amylase trypsin inhibitors, the proteinaceous components implicated in non-coeliac gluten sensitivity.

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Protein extraction is a critical step in attaining optimal results in proteomic studies. Total protein extracts from grains such as wheat, barley, rye and oats can contain non-protein compounds that may interfere with the analysis of the proteome. To temper the effects of interfering compounds, strategies such as defatting and/or protein precipitation are often employed. In this study we have used LC-MS/MS experiments comparing six total protein extraction protocols that employed different buffer compositions, with and without defatting and protein precipitation steps, in order to profile two cultivars of each of the gluten-containing grains. Using Tris-HCl and urea-based buffers 1433 and 1769 proteins were extracted from the barley cultivar Sloop respectively. Inclusion of a hexane-based defatting step prior to protein extraction was of no benefit, whilst protein precipitation employed post-extraction negatively impacted protein recovery, a likely result of problems associated with protein re-solubilisation. The use of an alcohol-based extraction protocol commonly used for enriching for gluten proteins also yielded a lower number (645) of proteins but was noted to co-extract the alpha-amylase/trypsin inhibitors (ATIs) that have been implicated as elicitors of the poorly characterised condition non-Coeliac gluten sensitivity. Using the discovery data collected, we developed multiple reaction monitoring (MRM) MS assays for the ATIs derived from wheat, barley, rye and oats. These methods were applied to the quantitative determination of the optimal extraction protocol for ATI enrichment. A modified two-step extraction method, i.e. alcohol-based extraction followed by urea buffer extraction was shown to yield the maximum level of ATIs. The level of ATIs was noted to vary between cultivars, with LC-MRM-MS analysis providing an opportunity to select grain varieties with differing levels of ATIs.

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Proteomic characterisation of stemness in rat bone marrow-derived mesenchymal stem cells

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Mesenchymal stem cells (MSCs) hold therapeutic potential for a number of pathologic conditions due to their ability to differentiate into various cell types. However, the clinical application of MSCs requires them to be maintained in a non-differentiated state, which remains a major challenge for the field. To develop ready-to-go MSCs, specific culture conditions that maintain the 'stemness' of the MSCs need to be formulated. In this study, the 'stemness' of rat MSCs cultured under six separate culture conditions is being investigated using a proteomics based approach.

Bone marrow derived rat MSCs were established in standard culture medium. Cells were then serum starved prior to culture for 72 hours in standard growth media supplemented with 2 % serum and a combination of fibronectin (FN), fibroblast growth factor 2 (FGF2) and/or bone morphogenetic protein 4 (BMP4). Cellular protein was collected and prepared using standard techniques for qualitative and quantitative (SWATH) mass spectrometry.

From this study, a library of all detectable proteins in rat MSCs was generated. Each protein was quantified across the culture conditions and the predominant biological processes were determined. Fibronectin was determined to have a significant role on the biology of the cells. Furthermore, statistical analysis of the quantitative profiles of each of the treatment groups revealed eight specific proteins that were of interest across the culture condition.

Characterisation of the rat MSC proteome provides insight into the molecular processes that occur within these cells while in a 'stem' state, allowing for the development of defined culture conditions that maintain MSCs in an undifferentiated state.

This study will provide much needed information on culture conditions that enable monitoring and maintenance of MSCs in a non-differentiated state, which in the future could permit an off-the-shelf MSC therapeutic to be developed for the treatment of a variety of medical conditions.

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SWATH analysis using external spectral libraries: Evaluation of cross-instrument data for library construction and quantitation

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Data independent mass spectrometry such as SWATH optimally requires a reference peptide MS/MS spectral library to link peak areas to identified peptides. Because the library needs to be comprehensive for discovery purposes and extensive data acquisition using the same mass spectrometer is not always possible, it is desirable and sometimes necessary to use LC-MS/MS data obtained from different mass spectrometers. We have previously described the SwathXtend bioinformatics tool to accommodate this application [1, 2].

In this presentation, we demonstrate the use of SwathXtend to process SWATH data from a TripleTOF 6600 (SCIEX) by merging a seed library to external libraries generated from a QExactive orbitrap mass spectrometer (Thermo Scientific) obtained in a different laboratory. We used 5 strains of *S. aureus* with 6 replicates grown in 2 different experimental conditions (n=60).

The quality of the process of library extension was evaluated using the reliable SWATH workflow described in [2] and was found to be good in terms of retention time correlations and relative ion intensity correlations. Additionally, the relative quantitation across the two platforms could be checked due to the controlled nature of the experiment in which the same samples were being run across the two instruments. This study demonstrated the feasibility for generating peptide MS/MS spectral libraries from two different MS architectures and using these for quantitation. The approach allowed quantitation of over 1300 proteins representing ~ 60% of the *S. aureus* predicted proteome.

[1] Wu JX *et al.*, Molecular & Cellular Proteomics, 2016, *15, 2501-2514*
[2] Wu JX *et al.*, *Proteomics* 2017, 17, 1700174, DOI: 10.1002/pmic.201700174.

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Integrated degradomics in differentiated macrophages

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Publish consent withheld

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Comparative Proteomic analysis using TMT-based MS Reveals New Insight between Different Growth Phase of Biofilms from *S. aureus*

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Introduction and Objectives

Staphylococcus aureus and coagulase-negative staphylococci comprises approximately 65% of infections associated with medical devices and are well known for their biofilm formatting ability. Currently, there is no efficient method for early biofilm detection. Our goal was to construct a reference map of planktonic and biofilm associated proteins of *S. aureus.*

Methods

S. aureus reference strain (ATCC 25923) was used to grow 24 hour planktonic, 3 day, and 12 day hydrated biofilms. Bacteria were grown in tryptic soy broth liquid medium. The Centres for Disease Control biofilm reactor was used to grow 3 day, and 12 day hydrated biofilms. Extraction and fractionation were performed using lysis buffer, ultra-membrane centrifugation, followed by reduction, alkylation and digestion steps prior to Multiplex labelling using Tandem Mass Tag (TMT) 10-plex reagent, respectively. TMT-based Mass spectrometry (MS) was performed and MS data was collected on an Orbitrap Elite Mass Spectrometer. Protein identification and relative quantitation of protein levels were performed using Proteome Discoverer (version 1.3). Biostatistical analysis was performed using the TMTPrePro R package.

Results and Discussion

We identified 1636 total secreted supernatant proteins, of which 350 and 137 proteins of 3DWB and 12DWB showed significant abundance variation from planktonic preparation, respectively. Of these, we found significantly up-regulated 59 and 71 unique proteins in this biofilm producer in between 3DWB and 12DWB, respectively. On the contrary, we found significantly down-regulated 74 and 48 unique proteins in this biofilm producer in between 3DWB and 12DWB, respectively. Therefore, a comprehensive knowledge of planktonic and biofilm associated proteins identified by *S. aureus* will provide a basis for future studies on the development of vaccines and diagnostic biomarkers.

Conclusions

In this study, we constructed an initial reference map of planktonic and various growth phase of biofilm associated proteins which might be helpful to diagnose biofilm associated infections.

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Unbiased Analysis of Liver Protein Complexes During the Fasting Response

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The liver is one of the largest and most important organs in the mammalian body with major functions in whole-body nutrient homeostasis. The response to nutrient deprivation (fasting/starvation) is a particularly important function for the liver. Regulation of hepatocyte signalling by circulating hormones, which can modulate protein-protein interactions, plays a critical role in this response.

To identify proteins that may regulate the liver fasting response by altering protein-protein interactions, we capitalised on the ability for size exclusion chromatography (SEC) separations to identify complex assembly and disassembly. We collected 48 SEC fractions per sample using two complementary SEC buffer systems and 3 biological replicates per condition (overnight fasted or fed). High sensitivity nanoLC-MS/MS analysis on a QExactive Plus and data analysis using the MaxQuant package, yielded >5,200 proteins identified and quantified across the biological replicates. After Pearson correlation analysis, we set thresholds for replicate profile variation (>0.75) and for inter-condition variation (<0.75). Less than 80 proteins showed a significant deviation in protein complex assembly/disassembly under these criteria that was not coupled to a simultaneous total protein abundance change.

One of the most interesting changes observed was for the protein phosphatase PP2A-B56 complex, containing the catalytic C subunit PPP2CA, the constant regulatory A subunit PPP2R1A and the variable regulatory B subunits of the B56-family. The PP2A-B56 complex displayed significant deviation in assembly of the main 300 kDa peak between fed and fasted animals. We have followed up these observed changes with immunoprecipitation of endogenous PP2A complexes from liver tissue for both proteome analysis and activity measurements in each condition. We hypothesise that this fasting-regulation of PP2A mediates control of the negative feedback this phosphatase provides directly on Akt, which forms a key element of the insulin signalling cascade in hepatocytes.

Deciphering the Pathways of Neuronal Excitotoxic Death by N-terminomics Analysis

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Excitotoxicity, over-stimulation of the extrasynaptic glutamate receptors is a pivotal neuronal cell death process underpinning brain damage in acute neurological conditions such as ischaemic stroke and chronic neurodegenerative diseases such as Alzheimer's disease. Glutamate, the main excitatory neurotransmitter of central nervous system initiates excitotoxicity by causing excessive influx of ions, mostly Ca^{2+} into neurons and resulting in the activation of different enzymes, such as calpains and caspases. These aberrantly activated enzymes then modify specific neuronal proteins that cause neuronal cell death. How these cellular proteins in neurons undergo proteolytic processing and eventually cause neuronal death, has not been clearly defined. To address this question we employed a high-content N-terminal positional proteomics technique, Terminal Amine Isotopic Labelling of Substrates (TAILS), to identify neuronal proteins that are post-translationally processed and modified in excitotoxicity. This approach involve stable isotope dimethyl labelling of the N-termini of proteins generated by limited proteolysis in primary cortical neurons after glutamate over-stimulation. In the experiment with glutamate treated neurons, we identified 63 proteins as potential key mediators in excitotoxic neuronal death. Further analysis of our study will unveil potential driver signalling pathways directing neuronal death in excitotoxicity.

An optimised size exclusion chromatography method enables analysis of the extracellular vesicle proteome from complex samples

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Extracellular vesicles (EVs) are membrane-bound nanoparticles containing protein and nucleic acids and are shed from cells into the surrounding environment. EVs are present in numerous circulating bodily fluids including blood, urine and saliva, and are thought to be a mechanism of cell-cell communication. Cancer cells appear to exploit this mechanism, constitutively releasing EVs to promote tumour growth and metastasis. As such, EVs are considered a promising minimally invasive biomarker source for cancer diagnosis and/or monitoring.

Proteomic analysis of EVs derived from complex biological fluids, such as serum or plasma, is challenging due to the co-isolation of highly abundant protein species such as serum albumin and apolipoproteins, with commonly used EV isolation methods.

In the current study, we aimed to optimise a method for size exclusion chromatography (SEC) purification of EVs from complex samples for downstream proteomics analysis. Whilst previous reports have demonstrated the potential of SEC for EV purification, the level of 'contaminating' plasma proteins that are co-isolated have not been evaluated. Using both a synthetic model system and real EV samples, we have demonstrated that both column size and resin composition have a substantial effect on the level of protein contamination, whilst the effect of mobile phase composition is negligible. These findings guided the development of an optimised workflow to purify EVs from healthy plasma and cell culture media samples and we were subsequently able to reduce the level of abundant plasma proteins detected by LC-MS/MS in purified EV samples. As proof-of-concept, a breast cancer cell line EV-specific protein signature could be detected when the EVs were spiked into healthy plasma, illustrating the utility of this method for preparing clinically relevant samples. We anticipate that this optimised method will accelerate future EV proteomics studies, enabling new insight into this promising source of protein biomarkers.

Next generation clinical veterinary proteomics

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Our work leverages the power of recent advancements in data acquisition strategies for mass spectrometry-based proteomics to drive clinical veterinary research into the next generation. It aims to establish well-curated peptide spectral libraries specific to proteins isolated from blood of multiple production animals (horse, cattle, sheep, goat) and build a Proteome Browser to link mass spectrometry data to genomic information and share this resource with scientific community. Once established, the Browser and associated tools will become a widely accessible on-line resource for archiving and decoding quantitative DIA profiles collected in ruminants that were acquired anywhere around the world and with different biological questions in mind. In our laboratories however, we specifically apply it to develop an array of plasma biomarkers to quantify the effectiveness of pain relief interventions during surgical husbandry procedures. In future, this resource could be expanded to other body fluids and tissues to advance veterinary medicine and agricultural research in a manner that has not been possible before.

Modelling multiple sclerosis: proteomic characterisation of adipose stem cells

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Multiple sclerosis is the most common cause of chronic neurological disability in young adults, despite this the aetiology is poorly understood and this is largely due to the limited availability of suitable tissue and limitations of current models. To gain insight on this complicated disease, a model was created which utilises adipose stem cells. This disease in a dish model compares adipose stem cells obtained from both multiple sclerosis and non-multiple sclerosis patients that have undergone neuronal differentiation. Cellular and secreted proteins were identified respectively by liquid chromatography tandem mass spectrometry and multiplex immunoassay respectively. This disease dish successfully recapitulated some aspects of the disease, and provided unique insight. The 27 cytokines that were examined are associated with immune signalling and contribute to neurological functions such as synaptic plasticity. As multiple sclerosis is an autoimmune disease, it is vital to understand the role inflammation has on the fate of stem cells. Cytokine expression in multiple sclerosis patients had greater homogeneity than that of non-multiple sclerosis patients, which was expected as the non-multiple sclerosis patient cohort could have various undiagnosed and undetectable health conditions. Interestingly, cytokine secretions significantly differed between patient cohorts and suggested that adipose stem cells isolated from multiple sclerosis patients have a reduced neurogenic capacity. This was supported through the analysis of cellular proteins as multiple sclerosis patient adipose stem cells had a reduced neurogenic capacity, and proteins associated with multiple sclerosis were identified. Phase contrast microscopy complemented this as the multiple sclerosis patient derived adipose stem cells did not appear to undergo the same morphological changes that are characteristic of differentiation. The data generated from this investigation offers a comprehensive analysis of adipose stem cell neurogenic capacity, and establishes a disease in a dish model that reveals disease insight.

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Novel capillary-flow LC HRAM MS platform for fast targeted analysis and robust profiling of complex samples

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Capillary flow LC-MS (capLC-MS) with 100-300 µm inner diameter (ID) columns and flow rates from 1 to 10 µL/min provide increased MS sensitivity, lower solvent consumption, and reduced MS contamination compared with typical analytical flow LC-MS .Here we present a highly robust novel capillary-flow LC-MS platform that combines the Thermo Scientific™ capillary-flow UltiMate™ 3000 RSLCnano system (capLC), the new 150 µm ID Thermo Scientific™ EASY-Spray™ column and the new Thermo Scientific™ Q Exactive™ HF-X mass spectrometer. Both shotgun and targeted proteomics experiments (Full-scan MS, Data-Dependent Acquisition (DDA), and Parallel-Reaction Monitoring (PRM)) were employed to verify the performance and robustness of the novel capLC-MS platform. We show that the novel capLC-MS platform is a sensitive and reliable solution for shotgun and targeted high-resolution accurate-mass (HRAM) proteomics experiments that can be used for routine proteome profiling of complex samples including bio-fluids as well as targeted high-throughput quantification.

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Characterisation of duck egg lysozyme isoforms by mass spectrometry

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Duck egg lysozyme (DEL) is a widely used model antigen owing to its capacity to bind with differential affinity to anti-chicken egg lysozyme antibodies. However, the use of DEL as a model protein has been complicated by the presence of multiple isoforms and conflicting reports of primary sequence.

In this study, three isoforms of DEL (DEL-I to DEL-III) were purified from Pekin duck (*Anas platyrhynchos*) eggs by carboxymethyl ion-exchange chromatography. In parallel with crystallography studies, the fractions were assessed by SDS-PAGE analysis and the three isoforms were shown to be well separated. The accurate intact mass of each the isoform was obtained by direct infusion on an Orbitrap Fusion mass spectrometer. Complementary sets of peptides were generated by proteolytic digestion with trypsin or Lys-C proteases. The peptides were then analysed either by MALDI TOF MS, using a QSTAR Elite QTOF mass spectrometer, or by Nanospray QTOF MS, using a 6600 QTOF mass spectrometer. By utilising multiple mass spectrometry approaches in conjunction with crystallographic data it was possible to report the non-ambiguous primary sequences for DEL-I to DEL-III.

Analysis of the primary sequences explains the sequential elution of the isoforms from the carboxymethyl resin. We were also able to resolve the identity of residues 66 and 103 which have been consistently problematic in their assignment in previously published reports. With the use of a protein-fold 'all-on-all' dendogram, it was now possible to relate the duck lysozymes to those found in other fowl, mammals, and animals.

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Spatial targeted metabolomics mapping in rat brain sections using DESI ion mobility mass spectrometry

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Spatial mapping of small molecules, such as neurotransmitters, alongside lipids, can increase our understanding of biological functions of those molecules within the brain. Mass spectrometry imaging (MSI) can be used to map distribution of molecules from any flat surfaces, including tissue sections. Desorption Electrospray Ionization (DESI) is an ambient ionization technique that can spatially profile the distribution of molecules in research tissue samples. DESI-MSI often provides complementary information to other techniques such as matrix-assisted laser desorption ionization (MALDI). Here we present the utility of DESI imaging to detect neurotransmitters, such as serotonin, adenosine, and glutamine directly in brain tissue samples. These results are for research use only and not for use in diagnostic procedures.

Rat brain was harvested and flash-frozen in liquid nitrogen before cryosectioning. Coronal tissue sections (8 microns thick) were mounted on regular glass microscope slides, vacuum dried, and analyzed without any further sample preparation. DESI-MSI data were collected and processed on a high definition mass spectrometer with ion mobility separation (SYNAPT HDMS G2-Si, QToF) using High Definition Imaging (HDI) 1.4 software with MassLynx 4.1 data acquisition control. DESI acquisitions were performed using methanol and water as a DESI spray solvent.

Small molecules such as amino acids (e.g., taurine, glutamine, arachidonic acid) and neurotransmitters (e.g., GABA, serotonin) were simultaneously detected along with lipids (e.g., phosphatidylcholine, lysophosphatidylcholine). Spatial correlation between detected metabolites and lipids were explored using analysis based on Pearson product-moment correlation coefficient and hierarchical clustering analysis. Molecular identification was aided using high mass accuracy (low PPM) database searches against publically curated databases, such as LipidMaps and Human Metabolome (HMDB). In addition to the accurate mass and high-fidelity isotopic distribution, collisional cross sections (CCS) or drift time data obtained during ion mobility separation was used to improve confidence in detected molecules.

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A multiplexed lipidomics study of the adaptive response of prostate cancer cells to androgen-targeted therapy

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The adaptive response of prostate cancer (PCa) cells undergoing androgen targeted therapies (ATT) were investigated. In this study, a long term *in vitro* ATT model was developed by treating LNCaP PCa cells with AR antagonist Enzalutamide (10 μM) for up to 21 days. Samples were collected at days 7, 14, and 21 of Enzalutamide treatment and compared to vehicle-treated LNCaP cells (DMSO control). High content imaging of fluorescent-labelled lipid probes demonstrate that PCa cells increase lipid uptake and remodel their lipid landscape as a response to ATTs. Interestingly, these ATT-treated cells show little proliferation and mitochondrial activity, suggesting that changes in lipid uptake and content, support pathways other than growth and oxidative phosphorylation. Lipidomics analyses of these cells was undertaken to give a more comprehensive understanding of the changes occurring at the molecular level. Shotgun lipidomics was performed by direct infusion of samples using loop injections on QTRAP 6500 (SCIEX, hybrid triple quadrupole / linear ion trap mass spectrometer). Samples were spiked with internal standards, SPLASH® Lipidomix® (Avanti polar Lipids) and fatty acid C19:0 during lipid extraction. Triple quadrupole functionality was used to perform specific precursor ion (PI) and neutral loss (NL) scans targeting numerous classes of phospholipids, glycerolipids and sterol esters. Data were combined and analysed using LipidView™ software (SCIEX) providing for lipid identification and quantification of 250 lipids at the sum composition level of identification. In parallel, lipid extracts from all samples were hydrolysed to release fatty acids which were then derivatized to the corresponding methyl ester using a trimethylsulfonium hydroxide reagent and analysed by GC-MS 8040 (Shimadzu 8040, triple quadrupole mass spectrometer). Changes in the absolute abundance of both intact complex lipids and fatty acids will be presented and discussed in the context of cellular responses to ATT.

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Absolute quantitation of iron binding proteins as therapeutic targets in neurodegeneration

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Iron is involved in many essential biological processes. Perturbed iron homeostasis, such as the accumulation of brain iron with age, can lead to oxidative stress and neuronal damage underlying neurodegenerative processes such as those observed in Alzheimer's disease. Ferritin is the protein responsible for safe iron storage, and is conserved across taxa, including *Caenorhabditis elegans*. This microscopic nematode is a widely used animal model of ageing and is easily genetically manipulated. The *C. elegans* genome is well characterised, and importantly has homology with higher-order species providing an opportunity to study the relationship between neurodegeneration and iron metabolism.

Here we present an optimized protocol for purification and absolute quantitation of ferritin from cell lysate using custom ([13]C- and ^{15}N-leucine/isoleucine) peptide standards, and parallel reaction monitoring (PRM)-based targeted mass spectrometry. Chromatographic separation was achieved within 60min ($\%RSD<2\%$), with 35% enrichment of ferritin signal from purified lysate. Linearity was established over 4 orders of magnitude ($R^2>0.99$), with instrument limits of detection <1 fg on column. Inter-assay reproducibility was ~30%RSD. This method will be applied to an aged *C. elegans* populations to observe changes in iron

homeostasis in ageing animals, before being transferred to other to other animal systems, such as the mouse. When conserved across taxa, these changes present a therapeutic target for age-related neurodegenerative diseases in humans.

Robust reproducible LC/MS based metabolomic profiling of large cohort human studies using validated high throughput targeted and discovery assays

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As global life-styles change we are seeing increasing cases of obesity, diabetes, and mental health issues. This not only affects a person's quality of life but also places increased strain on the health-care systems to provide the right treatment whilst managing costs closely.

Metabolic Profiling offers a valuable and unique insight into the underlying biochemistry of diseases as well as the patient's individual biochemistry "phenotype', diet, health status, age and stress. To deliver this information the analytical data generated in processed via a variety of chemometric modelling and analysis methodologies to deliver the relevant biochemical information. These chemometric platforms employed vary from simple multivariant analysis to highly complex model based analysis and is presented in a format ready for interpretation by medics.

In this presentation we will discuss the development of both exploratory and targeted metabolomics and lipidomic LC/MS analytical platforms as well as a detailed discussion on the workflow, validation, reporting and decision making process. The quantitative targeted LC/MS assays discussed will include as bile acids, amino acids, eicosanoids, and acyl cartanines, oxylipin, tryptophan metabolism and gut microbiome. The presentation will also cover the development and validation of high throughput, less than 2 minutes, "discovery' screening methods for polar, non-polar metabolites and lipid profiling using LC/MS methodology, as well as describe the use of Ion Mobility Mass Spectrometry to enhance data quality.

1. Ion mobility spectrometry combined with ultra performance liquid chromatography/mass spectrometry for metabolic phenotyping of urine: Effects of column length, gradient duration and ion mobility spectrometry on metabolite detection. Rainville, P.D., Wilson, I.D., Nicholson, J.K., Issacs, G., Mullin, L., Langridge, J.I., Plumb, R.S. Analytica Chimica Acta Volume 982, 22 August 2017, Pages 1-8
2. High-Speed Quantitative UPLC-MS Analysis of Multiple Amines in Human Plasma and Serum via Precolumn Derivatization with 6-Aminoquinolyl-N-hydroxysuccinimidyl Carbamate: Application to Acetaminophen-Induced Liver Failure. Gray, N, Zi, R., King, A., Patel, V.C., Wendon, J., McPhail, M.J.W., Coen, M., Plumb, R.S., Wilson, I.D, Nicholson, J.K. Analytical Chemistry Volume 89, Issue 4, 21 February 2017, Pages 2478-2487
3. Development of a Rapid Microbore Metabolic Profiling Ultraperformance Liquid Chromatography-Mass Spectrometry Approach for High-Throughput Phenotyping Studies. Gray, N, Adesina-Georgiadis, K, Chekmeneva, E, Plumb, R.S, Wilson, I.D., Nicholson, J.K. Analytical Chemistry Volume 88, Issue 11, 7 June 2016, Pages 5742-5751

Generation of rat organ SWATH-MS proteomic models for human cancer samples

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Each year, over 950 children and adolescents (0-19 year olds) in Australia – and 163,000 children worldwide – are diagnosed with cancer (1). The most common types of childhood cancers affect a range of tissues such as blood, lymph, bone marrow, nervous tissues, muscles, kidney, liver and bone. ProCan (The ACRF Centre for the Proteome of Human Cancer) is an industrial-scale proteomics facility at the Children's Medical Research Institute (CMRI). ProCan researchers have a major technological edge by combining pressure cycling technology with SWATH mass spectrometry (PCT-SWATH-MS) (2) that will be adopted to discover, verify and validate specific protein-based markers for many different types of cancers (from children to adults). To meet this goal, a major challenge is the generation of quantitatively accurate and highly reproducible data matrices of proteins with minimal missing values across cohorts of patients, whilst simultaneously accounting for variabilities among samples collected from different tissue origin.

Developing a SWATH-MS based profile of different normal tissues will provide an important reference to guide interpretation of cancer proteomic data. Specifically, it will assist in understanding aspects of tumour differentiation, and tissue origin in the case of metastatic disease. The study is designed to complement this aim by generating a rat tissue proteome using quantitative proteomics. In order to construct an organ specific quantitative proteome, PCT-SWATH-MS was applied to create specific spectral reference libraries of samples collected from seven different rat organs (Brain, Liver, Muscle, Spleen, Lung, Kidney and Testis). These results will be adopted for constructing a comprehensive proteomic profile for human tumour biopsies based on their site of origin.

Ref:
1. 0-19 years incidence data. Australian Institute of Health and Welfare (AIHW) Australian Cancer Incidence and Mortality (ACIM) Books   2017
2. Guo T et al (2015) Nature Med 21: 407 – 15.

Trapped ion mobility spectrometry with parallel accumulation serial fragmentation (PASEF): the new standard for shotgun proteomics

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Background: The "Parallel Accumulation - Serial Fragmentation" method (PASEF, Meier et al., JPR 2015, PMID: 26538118) for trapped ion mobility spectrometry (TIMS) coupled to a quadrupole time of flight (QTOF) instrument, has been described with the promise of achieving five to ten times faster data dependent acquisition of fragment ion spectra with improvements in sensitivity. Here we present results showing that the promise of PASEF has been realized.

Material & Methods: The performance of a timsTOF instrument with PASEF for shotgun proteomics has been evaluated by using tryptic digests of human cancer cell lysates (HeLa) spiked or not with an UPS peptide mixture, and separated by 90 min nanoLC gradients. Data were analyzed using DataAnalysis (Bruker), Mascot (www.matrixscience.com), and MaxQuant (Cox group, MPI of Biochemistry) or PEAKS (Bioinformatics Solutions).

Results: A standard 1.1 second PASEF acquisition cycle, which performs over 120 MS/MS at high sensitivity, identifies over 2900 protein groups from a 12 ng injection of a Hela cell digest separated with a 60 min gradient, and over 4800 protein groups from a 100 ng injection with the same gradient. In parallel, ion mobility separation allows to separate isobaric co-eluted peptides prior to fragmentation, further increasing the ID rate.

Conclusion: By enabling data dependent acquisition at very high speeds with improved sensitivity, the timsTOF Pro with PASEF enables researchers to dig deeper into the proteome, using less sample, yet finding more proteins of biological relevance.

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2D-precursor selection for trapped ion mobility with parallel accumulation serial fragmentation (PASEF)

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Background: With the previously introduced "Parallel Accumulation Serial Fragmentation" method (PASEF, Meier et al., JPR 2015, PMID: 26538118) for ion mobility (IMS) quadrupole time of flight (QTOF) instruments, five to ten times faster data dependent acquisition of fragment ion spectra became possible. This approach requires a fast, two-dimensional precursor selection algorithm, using mass as well as ion mobility information.

Methods: In PASEF mode, peptide ions elute from the IMS device as a condensed package. For most efficient MSMS acquisition, the quadrupole isolation window needs to switch its isolation position exactly synchronized to these elution times in the fastest possible order. The corresponding algorithm was developed and evaluated using tryptic digests of human HeLa cell lysates, separated by 90min nanoLC gradients. Data were analyzed using DataAnalysis(Bruker), Mascot(www.matrixscience.com), and MaxQuant (Cox group, MPI of Biochemistry).

Results: The precursor selection algorithm detects m/z and mobility positions of all precursors in the MS1-IMS scan. Then they are scheduled for measurement across multiple (ten to twenty) consecutive IMS experiments aiming for the most efficient utilization of measurement time. Low intensity precursors are measured multiple times to achieve sufficient spectra quality. Elution length of an individual precursor ion is dependent on IMS resolution, a function of mobility scan time. For tryptic peptides, mobility scan times of 25, 50, 100 and 200 ms resulted in average mobility resolutions between 20 and up to 80 and elution lengths between 1.8 and 10.6 ms. With a 100ms IMS separation up to 900 000 individual PASEF MSMS spectra can be acquired during a 90 min HeLa nanoLC-run, resulting in about 200 000 unique MSMS spectra after combining repetitive measurements.

Conclusion: We developed a 2D-precursor selection algorithm for PASEF that determines and schedules precursors in complex samples within 100-200 ms, well suited for an LC timescale.

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Quantitative analysis of differential protein distribution in brain regions using PCT-SWATH-MS

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Analysing the measurable proteome in complex tissue samples using mass spectrometry (MS) can be time-consuming with traditional methods. Pressure cycle technology (PCT) significantly reduces sample preparation time, and increases robustness and consistency. When coupled with SWATH-MS all detectable peptides in a sample can be quantified in a one single run. Our aim was to test the capabilities of PCT-SWATH-MS so see if the sensitivity could be compromised due to high acquisition speed. We tested the methods capability to quantify differential protein distribution in subregions within an body organ.

We chose to use five different brain regions, motor- and somatosensory cortex, hippocampus, amygdala and thalamus. Samples were collected in triplicate from each region with a biopsy punch from three different 24 week old Sprague Dawley rats. Samples were prepared in three different PCT instruments (Barocyclers) for homogenisation and tryptic digestion. The digests were run in duplicates on a Sciex TripleTOF 6600. The resultant SWATH-MS data were analysed and quantified using an internal rat brain spectral reference library (SRL) that was generated on the same type of instrument and HPLC gradient using standard shotgun methods. Data visualisation and sample QC was performed with Skyline, MarkerView, VennDIS and Panther.

The data showed that the fast 6 hour sample preparation from PCT did not comprise peptide concentration (2.5 µg peptides per µL) and consistency of digestion efficiency (80%). The SWATH-MS data identified about 3,000 proteins and 11,000 peptides in each sample, suggesting good depth. In MarkerView three protein clusters were observed: 1) motor- and somatosensory cortex, 2) amygdala and hippocampus, 3) thalamus. Each region had between 30 to 60 unique proteins visualised with VennDIS, with the somatosensory cortex having the highest amount of 62 unique proteins out of 2,856 proteins (2%). The unique proteins found in each brain region did not have common gene ontology pathways. Compared to traditional sample preparation and shotgun MS methods, PCT-SWATH-MS reduced sample preparation from traditional taking 12-24 hours to 5-6 hours and the label-free quantification reduced the cost significantly. The results show that PCT-SWATH-MS is a high throughput and reliable method for detection and label-free quantification of small differences in protein expression between different brain regions.

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Using quantitative proteomics to aid the diagnosis of mitochondrial disease and characterise novel disease genes.

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Mitochondrial disorders are clinically heterogeneous with causative mutations identified in over 250 genes. Whole exome sequencing has transformed diagnosis of these disorders but up to 50% of cases remain unsolved. This can be caused by failure to detect or interpret mutations in genes of interest and because some genomic regions remain refractory to analysis or interpretation. We aimed to identify novel mitochondrial disease genes and to exploit quantitative proteomic analyses to understand the underlying pathogenic mechanisms.

In one example, I will discuss how we identified large deletions in the *ATAD3* locus encoding 3 highly homologous tandemly arrayed genes (*ATAD3C, ATAD3B* and *ATAD3A*), their homology severely complicating genetic analyses. Subjects carrying the deletions suffer congenital pontocerebellar hypoplasia and carried similar deletions of ~38 kbp that resulted in an *ATAD3B/ATAD3A* fusion that produced a protein 99% identical to ATAD3A, but under the control of the ATAD3B promoter. Quantitative proteomics confirmed this decrease and showed that peptides unique to ATAD3B were found only in the N-terminal region.

In the second example, I will describe a novel mitoribosomal protein defect. The synthesis of all 13 mitochondrial DNA (mtDNA)-encoded protein subunits of the human respiratory chain is carried out by mitochondrial ribosomes (mitoribosomes). We discovered pathogenic mutations in the gene encoding the small mitoribosomal subunit protein, MRPS34. Subjects carrying the mutations suffered Leigh syndrome, and exhibited a combined mitochondrial respiratory chain defect due to impaired translation of mtDNA encoded subunits. Examination of the mitoribosome profile and quantitative proteomics showed the translation defect to be caused by destabilization of the small mitoribosomal subunit and impaired monosome assembly.

In conclusion, quantitative proteomic analyses of patient cell lines have much potential for identifying and proving causation in mitochondrial and other inherited metabolic diseases.

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Quantitative characterisation of burn blister fluid using SWATH to aid clinical diagnosis

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At present, the diagnosis of burn injury severity is largely dependent on the clinician's experience, which can limit timely intervention and subsequently scarring. The analysis of burn blister fluid (BF) presents an opportunity to aid in diagnosis, as biomolecules in BF reflect relevant systemic and local responses to injury. Our aim was to investigate burn injury proteomics using LC-MS/MS and to facilitate the development of quantitative measures that aid diagnosis to prevent adverse scarring outcomes.

In order to generate a comprehensive peptide spectral library, a subset of BF samples were pooled according to burn depth (12 superficial, 12 deep-partial thickness, and 4 full thickness) and fractionated by four different methods, including ultrafiltration, SDS-PAGE, OFFGel isoelectric focusing and immuno-depletion, prior to LC-MS/MS analysis in data-dependent acquisition mode. All individual BF samples (n=101) were then analysed using LC-MS/MS in data independent acquisition mode (SWATH) to obtain quantitative data.

More than 800 individual proteins were identified and formed the basis of a BF protein library. This protein library was further refined to a peptide ion library for SWATH data extraction. Therefore, the relative abundance of more than 600 proteins in every individual sample was obtained and correlated with different clinical parameters, such as burn depth and time to re-epithelialisation. Analysis of these data using statistical methods, including orthogonal partial least squares–discriminant analysis (OPLS-DA), ANOVA / t-test, and clustering analysis revealed the key biochemical differences that stratify sub-groups within the clinically relevant parameters.

We have shown that the blister fluid proteome can be used to classify paediatric burn wounds by different burn depths and by other clinically relevant parameters. The blister fluid proteomics could possibly predict the final burn depth at an earlier stage of the injury response. These markers are under further investigation to determine their viability as clinical diagnostic tools.

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Complete multi-omic physiological characterisation of clonal *Pseudomonas aeruginosa* adaptation to the CF lung

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One of the biggest challenges to quality of life for an individual with Cystic Fibrosis (CF) is the high rate of incidence of infection with the frequent isolate *Pseudomonas aeruginosa*. Infections are typically lifelong, resulting in significant morbidity and mortality, and lead to high rates of divergent within-host evolution, often resulting in the presence of multiple infection phenotypes. This renders traditional therapies / interventions ineffective. We profiled within-host adaptation by investigating a pair of isogenic clonal epidemic isolates (AUS-1E and AUS-1C), isolated from the same patient 11 years apart. Using a combined -omic strategy, we first investigated differences at the genomic level through long-read PacBio sequencing. Isolates were then grown in an artificial sputum-like medium that reflects the physiology of the CF lung. We undertook intra- and extra-cellular comparisons at the proteomics level using offline HILIC peptide fractionation coupled to reversed phase LC-MS/MS, which enabled the identification of functional clusters associated with virulence that were highly expressed only in the initial colonizing isolate. Cell surfaces of both isolates grown in artificial CF sputum medium were compared by MALDI-TOF MS of plate colonies, as well as glycolipids extracted following growth. We also investigated the intra- and extra-cellular lipidome. Differences in virulence capabilities were assessed through confocal scanning laser microscopy to investigate changes in biofilm structure, as well as virulence in the classical *C. elegans* slow-killing and murine lung-infection models. Host-pathogen interactions were investigated using 16-colour flow cytometry within the murine lung. The results obtained provide one of the most comprehensive assessments to date of the consequences of over a decade of within-host evolution on the cellular physiology of *P. aeruginosa* in adaptation and persistence within the context of CF.

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Analysis of the cardiac ubiquitinome in a rat model of type II diabetes mellitus

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Type II diabetes mellitus (DM) remains one of the major causes of morbidity and mortality in the industrialized world. Several animal and human studies have found reactive oxygen species (ROS) as one of the key mediators of signal transduction during DM and DM-mediated cardiovascular disease (CVD). In addition to redox-specific post-translational modifications (PTM), accumulating evidence suggests the involvement of ROS in accelerating non-specific ubiquitination of cardiac proteins as a label for protein degradation. Our group has defined changes in several protein PTMs in DM and DM-mediated CVD using Langendorff perfused rat hearts and begun to understand how these various PTMs cross-talk with each other. Here, we examined the ubiquitin-modified cardiac proteome in DM.

Rats were fed a standard chow citrate (CC) (12% fat) or high fat (HF) (42% fat) diet for 8 weeks, with DM induced in 50% of the animals after 4 weeks utilising a low dose of streptozotocin (STZ; 55mg/kg); a pancreatic β-cell toxin. At the cessation of the feeding protocol, animals were euthanised and hearts excised. Cardiac hemodynamic performance was measured in hearts subjected to non-ischemic perfusion. Myocardial protein lysates were subjected to Western blotting using anti-ubiquitin antibodies and peptides enriched via immunoprecipitation using anti-diGly remnant antibodies for analysis by LC-MS/MS. Western blotting revealed gross changes in ubiquitination across the different groups. We identified 2,043 and 1,575 unique ubiquitination sites, originating from 669 and 579 unique proteins in CC and HFDM, respectively. These modified proteins were mapped to functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, identifying enriched regulatory pathways such as metabolic, proteasome, glycolysis/gluconeogenesis, cardiac muscle contraction and dilated/hypertrophic cardiomyopathy pathways. Our data suggest that the rat cardiac ubiquitinome is sensitive to diet and DM, indicating a possible role for ubiquitination in this disease.

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A web-based quality control tool for the bench marking of proteomic LC-MS systems

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Liquid chromatography-Mass spectrometry (LC-MS) is a mature technology for high-throughput proteomics. The significance of quality control (QC) has been long-acknowledged as a prerequisite for proteomics investigation. Many research laboratories and core facilities implement QC measures as part of their routine workflows. Despite this, few existing QC tools have been developed to assess the performance of the LC-MS systems, and to detect poor or deteriorating performance. Of those that have, often they

have specific use cases, are not easily implemented or are not open to public data submission. In addition, different types of instruments have different levels of performance, so platform-dependent QC tests are required.

Here we present a standardized workflow for the rigorous assessment of LC-MS performance. It starts with running of 1 μg HeLa cell tryptic digest, using a defined LC-MS method. Raw data is processed using the MaxQuant computational platform using a consistently-defined method and database. A series of output files are uploaded to our web-based application to assess the performance of multiple parameters.

The interactive analysis output provides a historical report from the latest runs to as far back as database goes. Advanced options also allow users to interactively compare individual runs, versus those in the database, comparing twelve most important features associated with instrument performance. Unlike previous QC tools, our web-based interactive application does not require any specific set-up, and individual or multiple samples can be analyzed though our application. It also allows users to simultaneously visualize the computational analysis and quantitatively interpret their data while monitoring the performance of their instrument over time by comparing the database. The application will be available to the proteomic community to submit their own samples.

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Multi-Organ Proteomics Analysis of a Rodent Model of Type 2 Diabetes Mellitus

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The increasing prevalence of diabetes, in particular type 2 diabetes mellitus (T2DM), warrants improved understanding of the molecular mechanisms and adaptations of not only primary targets of damage, but also those associated with the progression of the disease. The clinical etiology of T2DM suggests that insulin resistance of peripheral tissues arising from an energetic imbalance and increased adiposity pre-dates pancreatic hyperinsulinemia, in an attempt to lower blood glucose levels in response to this resistance. These events proceed pancreatic exhaustion and eventual insufficiency and the characteristic elevated blood glucose levels associated with T2DM. Given the complexities associated with disease progression and the potential for whole body effects involving diverse target organs, we took a systems-wide approach to investigate proteome adaptations in response to T2DM. We used a rodent T2DM model that combines the effects of increased calorie-loading via a high-fat diet (HFD) and pancreatic insufficiency by injection with streptozotocin (STZ; HFD STZ) and compared this with associated feed control (CHOW) control groups (CHOW control, CHOW STZ, and HFD control). Using this model, we observed elevated BGL in both STZ groups (STZ control and HFD STZ) indicative of pancreatic insufficiency. Dyslipidemia was noted, with elevated serum triglycerides present in HFD treated groups (HFD control and HFD STZ). We harvested 7 different organs including adipose tissue, brain, heart, kidney, liver, pancreas and skeletal muscle from across the 4 biological groups and subjected each to a quantitative label-based proteomics workflow using offline HILIC fractionation coupled to RPLC-MS/MS on a Q-Exactive HF instrument. We mapped over 10,500 proteins across these organs and performed hierarchical clustering to observe which organs showed increased sensitivity to the individual effects of HFD, STZ and finally the combined HFD STZ. This approach showed that T2DM has both systems-wide and organ-specific effects that have not previously been considered in the pathogenesis of this disease.

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The Power of Multiplexing - Combining TMT discovery and targeted label free quantitation for biomarker analysis

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Introduction

Isobaric labeling techniques TMT have become popular for biomarker discovery due to higher throughput and better precision and accuracy. The next verification step (10-50 patients) is challenging when balancing the target numbers and devoted instrument time. Here, we propose a workflow for plasma proteomics from multiplexed TMT biomarker discovery to rapid and robust verification using capillary flow LC on a novel Orbitrap platform with up to 40Hz scan speed.

Methods

Plasma from normal and diabetic patients were depleted, digested, labeled with TMT six-plex reagents, mixed at 1:1 ratio, and fractionated. The fractions were separated in a 120min gradient followed by analysis on an Orbitrap instrument. Data were analyzed by Proteome Discoverer™2.2 software. For targeted analysis, the same depleted, but unlabeled, samples were separated at a flowrate of 2-5ul/min and analyzed using parallel reaction monitoring (PRM). The data were processed by Skyline or Spectronaut software.

Preliminary data

The multiplexing capability of TMT labeling significantly saved instrument time and provided possibilities to perform extensive fractionation. Fractionation, combined with the new depletion columns, made the detection of plasma proteins spanning to 5 orders of magnitude accessible.

Over 200 peptide targets, which showed significant difference (>2 fold change) between normal and disease states in the above discovery experiment, were selected for label free targeted quantitation using PRM. Retention time prediction of unlabeled

peptides was performed using adjusted hydrophobicity index calculations. LC separation at the capillary flow rate provided high sensitivity and offered improved retention time reproducibility and robustness. The fast scan speed on the new Orbitrap platform greatly facilitated the detection of hundreds of targets in a single experiment. This rapid and robust quantitation method confirmed the biomarker candidates found in isobaric labeled discovery experiment.

Conclusion

The workflows described here enable the biomarker discovery and validation in a highly multiplexed and rapid manner.

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Single amino acid resolution of glycosites with top-down UVPD of glycoproteins

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Introduction

Ultraviolet photodissociation (UVPD) is a powerful tool for top-down proteomics due to the high efficiency and indiscriminant nature of its fragmentation. While UVPD has been demonstrated for glycopeptide and glycan analysis, it has not yet been tested on intact glycoproteins. Here we demonstrate the utility of top-down UVPD for analyzing both the composition and locations of glycosylations

Method

Disulfide intact and reduced and alkylated glycoprotein ions were produced by static nanoelectrospray ionization from denaturing solutions of 49/50/1 water/methanol/acetic acid. Ions were analyzed on a Thermo Orbitrap Fusion Lumos Tribrid MS, and top-down fragmentation was performed with HCD, ETD, or ultraviolet photodissociation at 213 nm for each ion.

Results

UVPD fragmentation of disulfide reduced proteins produced predominantly a and x ions and preferential fragmentation at proline residues, consistent with previous observations for glycopeptides. UVPD of disulfide reduced ribonuclease B resulted in single amino acid resolution for the site of the glycan, and fragment ions were composed predominantly of cleavage along the protein backbone with retention of the entire glycan, whereas UVPD of disulfide intact ribonuclease B resulted in predominantly cleavage of the entire glycan from the precursor and charge reduced precursor ions, and close to 100% sequence coverage for the termini of the proteins up until the locations of the disulfide bonds. This data demonstrates that complementary information can be gained from top-down UVPD of folded and unfolded glycoproteins -- the exact masses of the glycans from UVPD of the folded form of the protein, and the residues on which those glycans are located from UVPD of the unfolded form of the protein.

Conclusion

This is the first demonstration of the power of UVPD for top-down analysis of glycoprotein

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In-Depth Characterization Of Intact Protein Standards Using Top Down Mass Spectrometry With Multiple MSMS Strategies

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Background

Complete and accurate characterization of intact proteins by mass spectrometry is possible today thanks to the latest technological development. We developed a standard mixture for LCMS quality control and method development. Here we present the top-down analysis of this mixture using various fragmentation techniques and MSn capabilities available on a modified Orbitrap Fusion Lumos Tribrid.

Methods

We performed direct infusion experiments and LC-MS experiments using a Pierce intact protein standard mix ranging from ~9 kDa to ~70kDa MW on an Orbitrap Fusion Lumos Tribrid MS modified with a 213 nm UVPD source and coupled with a Vanquish UHPLC system. Intact proteins were separated using 2.1 mm by 50 mm Acclaim columns with a 4 μm particle size. We performed CID, HCD, ETD, EThcD and UVPD fragmentation MSn experiments for structural investigation. Intact Protein spectra were deconvoluted with ReSpect or Xtract in Biopharma Finder 2.0. MS/MS spectra were analyzed with ProSightPC 4.1 and the ProSightPD node in Proteome Discoverer 2.1

Results and **Conclusions**

Optimal dissociation parameters were determined as a function of ProSight P score. It was determined, for example, that UVPD reaction times spanned from 15 to 50 ms for proteins ranging from 9 – 68 kD. ETD reaction times ranged from 20 to 10 ms for proteins ranging 9 – 68 kD To test consistency between infusion and LCMS, these optimized settings were translated to LCMS methods. These data not only confirms the translatability of these results and the value of these standards to optimize conditions during MS method development, but also intact protein LC method development and quality control. We will present here the range of optimized values in each fragmentation mode for proteins across the MW range, and discuss theoretical reasoning behind these experimentally determined settings.