OPENING SESSION

Taming glycoconjguate structure and interactions in development and disease

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Protein glycoconjugates, protein products modified with intricate glycan post-translational modifications, are often considered untamable biomolecules. A single glycoconjugate can exist in thousands of heterogeneous unique protein glycoforms in living cells, resulting in innumerable possible interactions and functions. Despite the structural diversity of glycoconjugates and the complexities of their interactions, there is information encoded in these molecular messages, such that they play key roles in orchestrating physiology and disease. However, current approaches to describe protein glycoconjugates and their interactions are limited, restraining our understanding of the important and often critical functions of protein glycoconjugates. Using innovative interdisciplinary approaches rooted in chemical biology and high resolution mass spectrometry, I will outline our efforts to dismantle the complexity of protein glycoconjugates. These endeavors include the fabrication of defined proteoglycans that regulate mouse embryonic stem cell differentiation, elucidation of the proteome-wide interactions of glycan-binding proteins in liver fibrosis and myogenesis, as well as the development of synthetic small molecules that differentially engage various protein glycoforms.

SESSION 01

Shear stress-dependent, non-canonical E-selectin ligands mediate tumor cell adhesion with low affinity and are targetable by proteasome inhibtion to prevent lung metastasis

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One bottleneck step of hematogenous metastasis formation of solid tumors is the dynamic endothelial adhesion of circulating tumor cells (CTCs). This process is mediated by E-selectin expressed at the luminal surface of endothelial cells and its carbohydrate ligands on tumor cells. In the lung microvasculature, E-selectin is induced by pro-inflammatory cytokines, which are systemically released from the primary tumors in the pre-metastatic phase. We observed that the cinically licensed proteasome inhibitor Bortezomib (Velcade) counteracts this cytokine-mediated induction of E-selectin in the lung, thereby reducing endothelial adhesion *in vitro* and *in situ* as well as lung metastasis formation *in vivo*. However, this novel anti-metastatic efficacy of Bortezomib was only detectable in case of tumor cells lacking the canonical E-selectin ligands sialyl-Lewis A and X (sLeA/X). Our data show that sLeA/X already mediate E-selectin binding and endothelial adhesion in the absence of shear force, obviously with such high affinity that the reduction in E-selectin achievable by bortezomib is not sufficient to impede adhesion/metastasis. Vice versa, we found that sLeA/X-negative tumors nevertheless adhere to endothelium in an E-selectin-dependent manner, so that non-canonical E-selectin ligands must exist that are still largely unknown. These ligands (e.g. glycoprotein-bound *N*-glycans on CD44) are shear force-dependent and confer such low affinity that the E-selectin-reducing effect achieved by Bortezomib is sufficient to reduce metastasis. Since sLeA/X are detectable by immunohistochemistry in biospecimens, patients could be stratified for an anti-adhesive, anti-metastatic therapy with Bortezomib during transient phases of increased CTC and cytokine release such as biopsies and surgical procedures.

1. Lange et al., Mol Ther 2022 Apr 6;30(4):1536-52.

Polysialic acid is dysregulated in the autoimmune disease scleroderma

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Scleroderma is a rare but devastating autoimmune disease that predominantly affects women. It is characterized by progressive immune dysregulation, organ fibrosis and vasculopathy and is the most lethal rheumatic disease, with no known cause and no curative therapies available. We discovered that polysialic acid (polySia) is dysregulated in patients with scleroderma. PolySia is normally limited to the immune, nervous, and reproductive systems of healthy human adults, where it plays pivotal roles in cell migration and attenuation of immune responses. We show that polySia is expressed in dermal fibroblasts (skin biopsies and cultured fibroblasts), but not healthy controls, and expression correlates with disease severity. This overexpression of polySia is due predominantly to expression of ST8Sia2 and is regulated by forkhead box 1 (FOXO1), a transcription factor that reduces oxidative stress. Our preliminary data suggested that disruption of polySia may decrease markers of disease, such as fibronectin expression, suggesting that polySia may represent a novel therapeutic target for scleroderma. We also measured serum polySia concentration and show that it is substantially elevated in patients with SSc and strongly correlates with disease progression in women but not men. We are investigating whether this increase in serum polySia is predictive and thus could be used to prognosticate the disease.

Immunological responses against glycosylated biotherapeutics and biodevices in humans

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Susceptibility to structural valve deterioration is one of the major drawbacks of bioprosthetic heart valves (BHVs). *N*-glycolylneuraminic acid (Neu5Gc) is an immunogenic dietary-carbohydrate antigen in humans because of inactivation of the gene encoding CMP-*N*-acetylneuraminic acid hydroxylase (*CMAH*), and all humans have circulating anti-Neu5Gc antibodies. We hypothesized that interaction of anti-Neu5Gc antibodies with Neu5Gc on BHVs could lead to immune response resulting in valve deterioration through calcification. We demonstrate Neu5Gc in both native calcified human valves as well as in calcified-BHVs, explanted from human patients, by HPLC and immunohistochemistry. Furthermore, anti-Neu5Gc IgGs were purified from native calcified human valves, validated by a glycan microarray. In the Neu5Gc-free *Cmah*-KO mouse model, anti-Neu5Gc antibodies promoted calcium deposits in subcutaneous implanted BHV discs, both with passive transfer of affinity-purified human anti-Neu5Gc IgGs, and by active-immunization of *Cmah*-KO mice with Neu5Gc-containing glyconanoparticles. Thus, co-existence of Neu5Gc/anti-Neu5Gc likely mediate BHV structural valve deterioration.

Spatiotemporal glycoprofiling of resting and maturing neutrophils reveals granule- and development stage-specific *N*-glycosylation

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Neutrophils are important innate immune cells equipped with an arsenal of glycoproteins packed in cytosolic granules that can readily be mobilised to elicit an effective, timely and well-balanced response against invading pathogens. While our knowledge of the intriguingly complex neutrophil glycobiology is rapidly expanding, important aspects of the structural complexity, spatial distribution, and temporal regulation of the heterogenous neutrophil glycoproteome remain undocumented. We here set out to comprehensively profile the highly complex and dynamic *N*-glycoproteome of mature (from blood) and immature (from bone marrow) neutrophils with spatial (granule fractionation) and temporal (maturation-stage separation) resolution. Using our quantitative LC-MS/MS-centric glycomics-assisted glycoproteomics approach, we firstly found that resting neutrophils exhibit granule-specific glycan signatures. In line with previous findings, the glycomics data demonstrated that the azurophilic granules display paucimannosidic *N*-glycans while the specific granules are dominated by complex *N*-glycans. The gelatinase granules and secretory vesicles exhibit complex- and oligomannose-type *N*-glycans, respectively. Importantly, the granule-specific glycoprofiles were recapitulated by the glycoproteomics data, which also revealed highly heterogenous site- and protein-specific *N*-glycosylation patterns decorating a total of 476 identified *N*-glycoproteins. To investigate the temporal remodelling of the glycoproteome during neutrophil maturation and the mechanisms driving the granule-specific glycosylation, we then reinterrogated publicly available LC-MS/MS proteomics and RNAseq transcriptomics datasets focusing on detecting previously overlooked glycopeptides and mining glyco-enzyme expression data, respectively, in discrete maturation stages of neutrophil precursor populations

isolated from bone marrow. Excitingly, we found that dramatic glycoproteome remodelling underpins the early promyelocytic-to-myelocyte maturation stage. Notably, our data indicated that the remodelling was driven predominantly by proteome expression changes as opposed to changes directly within the glycosylation machinery. Collectively, our findings provide new spatiotemporal insights into the immensely complex neutrophil *N*-glycoproteome, which exhibits fascinating site-, protein- and granule-specific *N*-glycosylation features and which undergoes strong *N*-glycoproteome remodelling during the early stages of neutrophil maturation.

Platelet glycans: take the bitter with the sweet

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Dr. Dianne van der Wal will present how removal of glycans, attached to the smallest blood cells, the platelets, especially sialic acid and neuraminidase affects their activation and clearance. She will discuss the role of platelet adhesion receptor GPIb and how desialylation by specific platelet antibodies is linked to platelet clearance in a bleeding disorder. Dr van der Wal will also discuss how some donor attributes effect surface glycans and quality of platelet components and implications for platelet clearance and hemostatic efficiency post-transfusion. She will also discuss a novel in vitro clearance glycan-mediated pathway for platelet released microparticles, and how this is linked to coagulation, and the potential implications for patients, post-transfusion.

SESSION 02

Deciphering the role of selective N-glycosyl modification in fate biasing of melanocytes from Neural crest cells

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The ability of multipotent cells to differentiate into distinct cell types is critical for vertebrate development. Pigment cell lineages delineate from the Neural crest cell (NCC) population and involve a complex gene regulatory network that is still not clearly understood. Understanding these cell fate decisions can be beneficial in deciphering the etiology of various degenerative disorders of melanocyte development such as vitiligo and melanoma. We performed a meta-analysis of Zebrafish single-cell RNA seq data to understand the molecular cues that drive the differentiation of NCCs to melanocytes. We observed that glucosyltransferase encoding gene MGAT4B was preferentially expressed in melanophores compared to other pigment progenitor cell types that arise from NCCs. To investigate the role of MGAT4B during the differentiation of melanocytes from NCCs, we silenced MGAT4B in the Zebrafish model system which led to a delay in melanocyte migration and an overall decrease in pigmentation levels. Furthermore, CRISPR/Cas9-based melanocyte-specific mutagenesis of MGAT4B in Zebrafish led to aberrant migration patterns and subsequent death of misguided melanophores. We also observed that MGAT4B expression was elevated in tumor biopsies of melanoma patients whereas, its expression was significantly reduced in vitiligo patients. Our results show that MGAT4B plays a central role in melanocyte development and needs further elucidation of its targets that may open therapeutic avenues in disorders of melanocyte development such as vitiligo and melanoma.

The SARS-CoV-2 S glycan shield evolution: Implications in immune evasion and viral infection along the phylogeny and VoCs

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Viral fusion proteins evade immune recognition by coating their surface with a dense cover of N-glycans, known as glycan shield[1]. Here, I will present how high-performance computing (HPC) molecular simulations have contributed to advance our knowledge on the role of glycosylation in the SARS-CoV-2 infection mechanisms. In this contribution, I will focus in particular on how the newly discovered functional role of the glycan shield in the S mechanism of action[2], in addition to its known role in immune evasion, makes it prone to evolutionary changes improving its host adaptation[3]. Indeed, important changes in the shield's topology have occurred along the SARS-CoV-2 phylogeny[3], representing a critical distinguishing feature between the SARS-CoV-2 S and SARS-CoV S, and zoonotic strains. I will also discuss how our simulations in combination with glycoproteomics describe the effects of new glycosylation sites that appeared on the S of the recent variant of concern (VoC) gamma, and how these could be linked to gamma's higher fitness relative to the then circulating VoCs[4].

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From MEMS to Carbon Dots: When Glyco meets Nano

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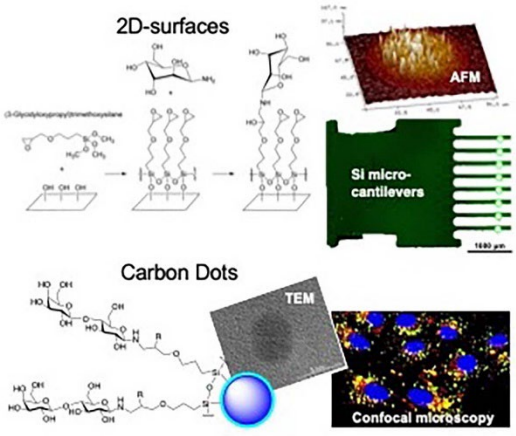
Micro-technologies in the form of Micro-Electro-Mechanical Systems (MEMS) and micro-plasmonics platforms offer the potential for high-resolution, high-throughput label-free sensing of biological and chemical analytes. Silicon carbide (SiC) is an ideal material for augmenting both MEMS and plasmonics routes, however such inorganic surfaces need to appropriately and efficiently functionalised to allow subsequent immobilization of functional biomolecules. To this end we trialed various organosilane-based self-assembled monolayers for the covalent functionalization of 2-dimensional surfaces including SiC films and Si micro-cantilevers, and have now developed an affordable, facile one-step method. Using high-throughput glycan arrays as our model system a novel platform that has the potential to combine established array technology with the label-free capabilities of MEMS or plasmonic systems is one step closer [1].

Using a similar functionalisation route, we have extended the use of organosilanes to biofunctionalise the surface of zero-dimensional nanoparticles, specifically carbon dots. Carbon dots are biocompatible, chemically stable, heavy-metal free quantum dots, of low toxicity that offer an alternative approach for bio-imaging and -sensing applications. Again, employing glycans as our model system, we have now used our biofunctionalization approach to generate glycan-coated carbon dots to explore complex glyco-interactions [2].



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A glycocode for the dynamics of breast cancer migration

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Carcinogenesis is a process in which frameworks of interactions between untransformed cells and their surroundings are subverted and replaced by new frameworks that seek to drive canonical behaviors of cancer cells. One such behavior is their tendency to migrate through surrounding extracellular matrices in a bid to spread to other organs of the body, a process known as metastasis. Migrations can be

complex and of distinct types, and are functions of the genetic and epigenetic aberrations that contribute to the new interactive frameworks associated with cancer cells. Alterations in the levels of glycans and their binding proteins, lectins are among the oldest and ubiquitous epigenetic changes in oncology.

I will showcase how we have built a multiscale computational model that predicts the structure of such frameworks, as well as the diversity of cancer migration modes. I will then discuss two short stories from my group, where alterations in levels of the galactoside binding protein Galectin-9 on the one hand, and intercellular heterogeneity in levels of 2,6-linked sialic acids on the other, drive distinct modes of migration of triple negative breast cancer cells. Our mechanistic findings reveal glimpses of a glycocode that could underlie the biophysical phase transitions in the migratory behavior of breast cancer.

Identifying the Regulatory Network of O-GlcNAc-Cyclers

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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 nuclear, cytoplasmic, and mitochondrial proteins by monosaccharides of O-linked β-N-acetylglucosamine (O-GlcNAc). Acute enhancement of O-GlcNAc reduces apoptosis and necrosis in models of injury that include oxidative stress and myocardial ischemia reperfusion (I/R) injury. To date, most of the work studying the O-GlcNAc modification in models of environmental and physiological stress has focused on identifying the proteins and mechanisms by which O-GlcNAc mediates cytoprotection. These studies have provided critical insight into the impact of O-GlcNAc on translation, molecular chaperone expression, metabolism, mitochondrial function, signal transduction, stress granule formation, and autophagy. We report here our efforts to understand the regulation of the enzymes that cycle O-GlcNAc in models of oxidative stress and myocardial I/R injury. In both cell culture and animal models, cellular O-GlcNAcylation appears uncoupled from the specific activity of the O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), the enzymes that catalyze the addition and removal of O-GlcNAc respectively. These data suggest that post-translational modifications and protein interactors of OGT and OGA control localization and substrate targeting resulting in protein-specific changes in O-GlcNAc in response to different stimuli. Our studies have begun to delineate both the OGT and OGA interactomes and how these regulatory networks are remodeled in response to injury, identifying at least one protein inhibitor of OGA – Fatty Acid Synthase.

SESSION 03

Ultra-sensitive platelet proteome maps the O-glycosylation landscape and charts the response to thrombin dosage

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Platelet activation induces the secretion of proteins that promote platelet aggregation and inflammation. However, detailed analysis of the released platelet proteome is hampered by platelets' tendency to pre-activate during their isolation and a lack of sensitive protocols for low abundance releasate analysis. Here we detail the most sensitive analysis to date of the platelet releasate proteome with the detection of >1,300 proteins. Unbiased scanning for post-translational modifications within releasate proteins highlighted O-glycosylation as being a major component. For the first time, we detected O-fucosylation on previously uncharacterised sites including multimerin-1 (MMRN1), a major alpha granule protein that supports platelet adhesion to collagen and is a carrier for platelet factor V. The N-terminal EMI domain of MMRN1, a key site for protein-protein interaction, was fucosylated at a conserved threonine within a new consensus sequence. The fucosyltransferase POFUT1 was subsequently identified as controlling MMRN1 secretion, supporting a key role of fucosylation in MMRN1 function. By comparing releasates from resting and thrombin-treated platelets, 202 proteins were found to be significantly released after high-dose thrombin stimulation. Complementary quantification of the platelet lysates identified >3,800 proteins, which confirmed the platelet origin of releasate proteins by anti-correlation analysis. Low-dose thrombin treatment yielded a smaller subset of significantly regulated proteins with fewer secretory pathway enzymes. The comprehensive platelet proteome resource provided here (larancelab.com/platelet-proteome) allows identification of novel regulatory mechanisms for drug targeting to address errors in platelet function and thrombosis.

Systems biology glycoproteomics, proteomics and metabolomics to investigate beer and wine production

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Beer is one of the most popular beverages worldwide. As a product of variable agricultural ingredients and processes, beer has high molecular complexity. We have used integrated glycoproteomics, proteomics, metabolomics, and genomics to investigate the molecular complexity and diversity throughout the beer making process. We uncovered substantial variability in barley based on variety and growth environment, and found that proteolysis during the mashing stage of beer production controls protein thermal stability and abundance in the final beer. Profiling 23 commercial Australian beers identified a very high diversity of post-translational modifications (PTMs), especially proteolysis, glycation of barley proteins, and glycosylation of yeast proteins. The key differentiator of the beer glyco/proteome was the brewery, with beer from independent breweries having a distinct profile to beer from multinational breweries. Within a given brewery, beer styles had distinct glyco/proteomes, with proteins in darker beers having low glycation and high proteolysis. Quantitative quality metrics of foam formation and stability correlated with the concentration of abundant surface-active proteins from barley and yeast. Finally, to expand the flavour and sensory profiles after fermentation we have used proteomics, genomics, and metabolomics to investigate wild yeast ferments for controlled production of diverse beer styles.

The glycome: The missing link in extracellular vesicle research

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Extracellular vesicles are naturally occurring nanoparticles that are released by all cells and serve important roles in physiological and pathological intercellular communication. Extracellular vesicles have promising applications as therapeutic agents, drug targets, delivery vehicles, and biomarkers. However, research progress is hindered by an incomplete understanding of the molecular composition of extracellular vesicles. In particular, the glycocode remains largely unknown due to technical challenges in extracellular vesicle isolation coupled with high-throughput glycomics. This talk will highlight a glycomics approach to understanding extracellular vesicles with a focus on breast cancer metastasis.

Contextual Tissue Cytometry Using AI-Empowered Precision Microscopy

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INTRODUCTION: In September 2021 the United States' Food and Drug Administration (US-FDA) has approved the first AI-based decision support system for prostate cancer diagnostics. This hallmark indicates a historic decision as it is the first time in the history of medicine that a regulatory body has accepted a software-only solution, which analyses microscopic images by using artificial intelligence!

As we move into the era of next-generation digital pathology, also referred to as computational pathology, current challenges in pathobiology comprise (i) automation of biomarker profiling in histological samples, (ii) quantitative integration of protein expression profiles and genetic information, as well as (iii) correlation analysis and data mining of high-plex data in-situ.

METHODS: Our research teams at TissueGnostics and Queensland University of Technology have joined forces to combine TissueGnostics' existing tissue cytometry technology platform and established knowhow with innovative AI solutions to establish The Virtual Histopathologist1.

RESULTS: Tissue Cytometry permits to determine the in-situ phenotype of cells as well as histological entities, like glands, vessels or tumor foci. Applications include but are not limited to the exploration of the tumor microenvironment and/or the spatial organization of cellular subpopulations, assessment of different bone structures, quantification of blood vessels and neovascularization as well as analysis of samples in multiplexing or multispectral mode.

Earlier attempts to analyse single cells in tissue have mostly been subject to visual estimation, or – at best – to manual counting for decades. To better understand the function of inflammatory cells in tumor development, type and number of inflammatory cells and their proximity to glandular/tumor structures have to be analyzed in-situ and correlated with disease state. Using TissueFAXS™ Cytometry the time-consuming and error-prone human evaluation of stained histological sections can be approached with an observer-independent and reproducible technology platform, offering a high degree of automation, paired with user interaction at relevant points of the analytical workflow (Fig. 1).

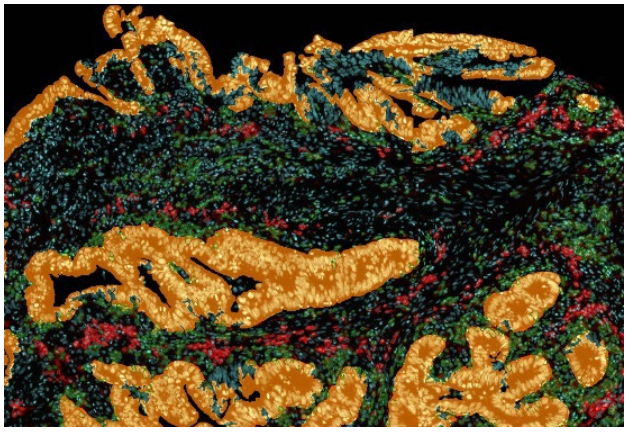

Fig. 1: Spatial immune-phenotyping in-situ. CD4+ T-Helper Cells in a distance of 25 to 50 µm to adjacent tumor (orange).

DISCUSSION & CONCLUSIONS: The TissueFAXS Cytometry platform incorporates Machine & Deep Learning algorithms. It can do end-point assays as well as live-cell imaging and time-kinetic experiments to measure enzyme activity. It also promotes tissue cytometry to a new level of quality, where complex cellular interactions, intracellular expression profiles and signal transduction cascades can be addressed on the single-cell level but still in histological context, empowering precision diagnostics.

ACKNOWLEDGEMENTS: This project was supported by three EU-funded Marie Skłodowska Curie Innovative Training Networks: (i) AIDPATH, (ii) CaSR Biomedicine, and (iii) HELICAL.

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Prostate Cancer Risk-Associated Single-Nucleotide Polymorphism Affects Prostate-Specific Antigen Glycosylation and Its Function

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Genetic association studies have reported single-nucleotide polymorphisms (SNPs) at chromosome 19q13.3 to be associated with prostate cancer (PCa) risk. Recently, the rs61752561 SNP (Asp84Asn substitution) in exon 3 of the kallikrein-related peptidase 3 (KLK3) gene encoding prostate-specific antigen (PSA) was reported to be strongly associated with PCa risk (P = 2.3 × 10-8). However, the biological contribution of the rs61752561 SNP to PCa risk has not been elucidated. Methods: Recombinant PSA protein variants were generated to assess the SNP-mediated biochemical changes by stability and substrate activity assays. PC3 cell-PSA overexpression models were established to evaluate the effect of the SNP on PCa pathogenesis. Genotype-specific correlation of the SNP with total PSA (tPSA) concentrations and free/total (F/T) PSA ratio were determined from serum samples. Results: Functional analysis showed that the rs61752561 SNP affects PSA stability and structural conformation and creates an extra glycosylation site. This PSA variant had reduced enzymatic activity and the ability to stimulate proliferation and migration of PCa cells. Interestingly, the minor allele is associated with lower tPSA concentrations and high F/T PSA ratio in serum samples, indicating that the amino acid substitution may affect PSA immunoreactivity to the antibodies used in the clinical immunoassays. Conclusions: The rs61752561 SNP appears to have a potential role in PCa pathogenesis by changing the glycosylation, protein stability, and PSA activity and may also affect the clinically measured F/T PSA ratio. Accounting for these effects on tPSA concentration and F/T PSA ratio may help to improve the accuracy of the current PSA test

SESSION 04

The role of glycosaminoglycans and their degrading enzymes in inflammatory cell homeostasis

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Serglycin is a proteoglycan (PG) present within the α-granules of mast cells, that has important roles in granule homeostasis by binding to and controlling the structure and activities of mast cell chemokines. Mast cell serglycin is predominantly decorated with the glycosaminoglycans (GAGs) heparin, and chondroitin sulfate (CS). Heparanase, a heparin degrading enzyme, present within the lysosomes and α-granules processes the structure of these GAG chains by reducing the molecular weight but an equivalent CS-degrading enzyme produced by mast cells has not previously been reported. This study investigates the production of the CS degrading enzyme hyaluronidase-4 (HYAL4) by mast cells and shows that HYAL4 is capable of cleaving the CS chains of CSPGs and serglycin into oligosaccharides ranging from tetra- to dodeca-saccharides in length. Mast cells were identified in human skin using histochemistry, and immunohistochemically with an anti-tryptase antibody. Flow cytometry and Western blot analysis demonstrated the presence of HYAL4 in mast cell lysates. Interestingly, flow cytometry analysis of mast cells revealed the presence of unique CS structures, detected with the monoclonal antibody 2B6, well characterized for the ability to bind to CS stub neoepitopes after digestion with the bacterial lyase, chondroitinase ABC. Treatment of CSPGs with HYAL4 generated structures detected with the CS stub neoepitope antibodies 2B6 and 3B3. These data demonstrate for the first time the production of a CS-degrading enzyme produced by mast cells, that may play a role in maintaining α-granule homeostasis by processing GAG chains into lower molecular weight forms.

The green challenge: methods to characterise the most complex polysaccharides on the planet

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Photosynthetic organisms are not usually carbon limited. As a result, the types and roles of glycans are hugely expanded in both structure and function. Plant glycans also shape human agriculture, manufacturing and diet, and form the basis of the emerging bioeconomy. However, analysis of these polysaccharides is challenging, and requires bespoke solutions. Here I will introduce two methods commonly used in my lab for the structural analysis of plant glycans: PACE (polysaccharide analysis by carbohydrate electrophoresis) and multi-dimensional solid-state NMR. PACE is a simple, cheap, and robust gel-based method that can provide separation of structural isomers at femtomole sensitivities. NMR, by contrast, is complex and expensive, but allows us to explore the intact 3-dimensional structure of polysaccharides in the cell wall, and explore functionality that derives from interactions. I will describe how we use both methods, and how combining these techniques is giving us new insights into glycan synthesis and function.

SESSION 05

Glycan Arrays, Diagnostics and Mycobacteriophages

Todd Lowary[1]
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Mycobacteria produce a range of different glycans that play important roles in modulating the host immune response that occurs upon infection. Understanding, at the molecular level, the protein–glycan interactions mediating these responses is poorly understood. This presentation will describe the use of an array of syntMycobacteria produce a range of different glycans that play important roles in modulating the host immune response that occurs upon infection. Understanding, at the molecular level, the protein–glycan interactions mediating these responses is poorly understood. This presentation will describe the use of an array of synthetic glycans to facilitate understanding the molecular basis of glycan-mediated immunity in mycobacterial disease, in particular, the specificity of antibodies that recognize the polysaccharide lipoarabinomannan. These insights have impacted the field of TB diagnostics and led to efforts to identify novel mycobacteriophage proteins that bind these glycans. hetic glycans to facilitate understanding the molecular basis of glycan-mediated immunity in mycobacterial disease, in particular, the specificity of antibodies that recognize the polysaccharide lipoarabinomannan. These insights have impacted the field of TB diagnostics and led to efforts to identify novel mycobacteriophage proteins that bind these glycans.

Probing the Effects of Glycosylation on Peptide and Protein Activity Through Chemical Synthesis

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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. Glycosylation has been shown to mediate an array of biological recognition events in all domains of life. Additionally, a number of recently approved biopharmaceuticals contain carbohydrate chains (or carbohydrate mimics) that are critical for activity and/or stability.[1] The non-templated enzymatic nature of the glycosylation process leads to heterogeneous mixtures of isoforms when glycopeptides and glycoproteins are isolated or produced in recombinant expression systems, thus hindering the ability to study how glycosylation influences 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. Our lab has recently developed a number of synthetic technologies to access homogeneously glycosylated peptides and proteins for structure-function studies and to assess the potential of these molecules as therapeutic candidates.[2,3] This talk will highlight the synthesis and evaluation of glycopeptide and glycoprotein hormones[4] and flagellin glycoproteins from Gram negative bacteria.

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Synthesis of Uronic Acid 1-Azasugars as Putative Inhibitors of α-Iduronidase, β-Glucuronidase and Heparanase

Gareth G Doherty[1], Geraldine G.M. Ler[1], Norbert Wimmer[1], Paul V Bernhardt[1], Roger Ashmus[2], David Vocadlo[2], Zach Armstrong[3], Gideon J Davies[3], Marco Maccarana[4], Jin-ping Li[4], Yasmin Kayal[5], Vito Ferro[1]

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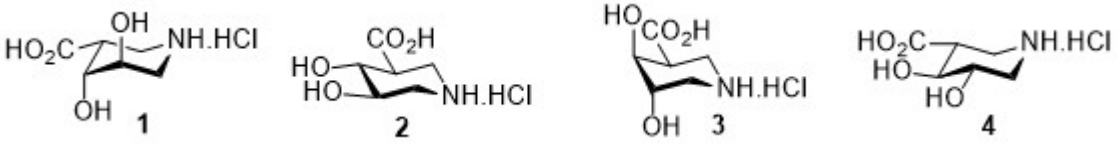
α-L-Iduronidase (IDUA), β-glucuronidase (GUSB) and heparanase (HPSE) are glycosidases that hydrolyze uronic acid residues in glycosaminoglycans such as heparan sulfate and dermatan sulfate. Deficiencies of IDUA or GUSB activity caused by genetic mutations lead to the lysosomal storage disorders MPS I or MPS VII, respectively. On the other hand, overexpression of HPSE is associated with a wide variety of diseases such as cancer, inflammation and viral infections. The synthesis of specific inhibitors of these glycosidases is thus of great interest for the development of new therapeutics. Herein we describe the efficient synthesis of 1-azasugars **1** and **2** from D-arabinose, and their corresponding enantiomers **3** and **4** from L-arabinose, as well as their evaluation as inhibitors of IDUA, GUSB and HPSE.



Towards Rapid Synthesis of Heparan Sulfate Mimicking Oligomers Through Peptide Chemistry

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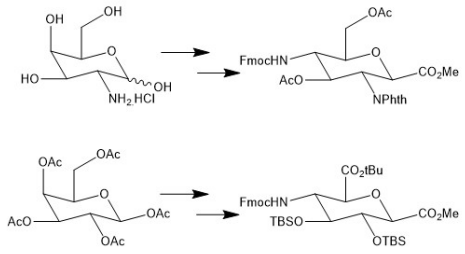
*2. Institute of Molecular Biology, A*STAR, Singapore*

3. School of Chemical Engineering, University of Queensland, Brisbane, Australia

Heparan sulfate (HS) is a glucosaminoglycan (GAG) polymer that is intrinsic to cell function and implicated in a myriad of biological processes. HS is a highly complex structure, largely comprised of a glucuronic-acid (1-4) linked glucosamine repeating backbone moiety. Huge dispersity is observed in chain length in this biopolymer as well as multi-site *O*-sulfation, *N*-sulfation and *N*-acetylation.

The chemical synthesis of HS oligomers, as a mechanism to probe activity *in vivo*, is long and time consuming. In this work we aim to produce a series of monomers as building blocks for the generation of structurally defined biopolymer mimetics that replicate the biological activity of HS-fractions we have derived from natural HS.[1,2] This methodology will permit the preparation of an HS-mimetic library utilising processes analogous to peptide chemistry.



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Understanding C-mannosylation-induced protein stabilisation through chemical protein synthesis.

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Tryptophan C-mannosylation plays an important role stabilising the fold of some proteins. The mechanism and degree to which it stabilises these proteins is not well understood, mostly because it has not been possible to obtain homogenous glycoforms for rigorous quantitative studies. To address this problem, we have developed sophisticated chemical methods to synthesise C-mannosylated peptide fragments, which can be used to assemble full-length proteins by native chemical ligation, then folded, to given chemically homogenous glycoprotein samples. We collected data for the thermal stability and thermodynamics of folding for an array of natural and unnatural glycoforms to demonstrate that not all C-mannosylation sites impact protein stability to the same degree. When coupled with molecular dynamics simulations, these data provided insights into the mechanisms underpinning tryptophan C-mannosylation-mediated protein stabilisation.

SESSION 06

Enhancing glycan imaging using photocleavable mass-tagged lectins for MALDI-MSI

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N-linked and O-linked glycans are the most common types of protein glycosylation in the human body and are involved in many fundamental cellular processes including protein folding, stability, and cell-cell interaction. These glycans are also extensively involved in the pathophysiological progression of cancer, from early changes in the tumour microenvironment, to metastasis, and immune system evasion. MALDI-TOF mass spectrometry imaging (MALDI-MSI) using specific enzymatic cleavage of N-linked glycans provides an ideal means to investigate the spatial distribution of N-linked glycans across complex biological samples as it is a relatively quick and easy technique. However, a major limitation currently is the lack of an enzyme to specifically cleave O-glycans, and therefore an inability to detect these glycans using the current MALDI-MSI methodology. Photocleavable mass-tagged (PC-MT) antibodies have been an exciting addition to the MALDI-MSI arsenal as they overcome many limitations of fluorescent probes. Unfortunately, the generation of antibodies against glycans is very difficult as they are seldom immunogenic and struggle to elicit high-affinity antibody production. Lectins are proteins that bind glycan moieties with high specificity and can be conjugated to various probes for histochemical analysis. The conjugation of PC-MTs to known lectins allows for the glycan specificity of lectins to be combined with the multiplexing capability of PC-MTs, enabling glycans that are difficult to detect (including O-linked glycans) and/or low abundance, to be visualized readily using MALDI-MSI. The aim of this study is to demonstrate the advantages of using PC-MT lectins to elucidate the spatial distribution of glycans in human ovarian cancer and combine this information with native N-glycan imaging, immunofluorescence, and optical imaging. Our hypothesis is that using PC-MT lectins for glycan imaging with MALDI-MSI will reveal the distribution of glycans integral in the pathogenesis of human ovarian cancer, novel diagnostic biomarkers, as well as potential therapeutic drug targets.

Novel Siglec Targeting Using Multivalent Sialyllactose Decorated Carbon Dots

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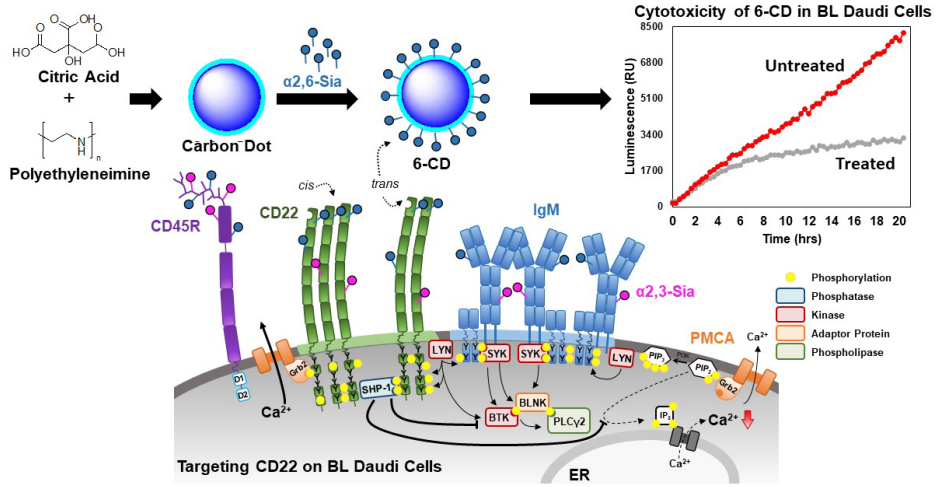
Sialic acid binding immunoglobulin-like lectins (Siglecs) are a family of receptors that regulate the innate and adaptive immune systems through glycan mediated signalling. Siglec ligand recognition has also been shown to be crucial for cell adhesion, cell signaling and endocytosis making them attractive targets for drug design. To this end, numerous high affinity synthetic sialoside ligands have been developed to serve as therapeutics for various Siglec driven disease states, however the role of multivalent Siglec ligands has not been studied in detail. Recently, advances in glyco-nanotechnology have allowed the development of multivalent scaffolds to explore carbohydrate-lectin interactions.[1] Of these, carbon dots (CDs), which are inherently fluorescent and have high surface/volume ratios, represent promising scaffolds to explore *in vitro* and *in vivo* glycan-Siglec interactions. Using our published synthesis route,[2] high affinity *α*(2,3)-sialyllactose (2,3-SL) and *α*(2,6)-sialyllactose (2,6-SL) decorated CDs (3-CD and 6-CD, respectively) to target Siglec-2 (CD22) and Siglec-1 (Sialoadhesin) were fabricated. We outline the use of SL-conjugated CDs in biology and show that they have an inherently high affinity to bind to siglec due to their multivalent display. Finally, for 6-CD, this multivalent display of Sia, was able to generate a significant cytotoxic effect on Burkitt's lymphoma (BL) Daudi B cells *(see figure 1)*. This study provides the framework for the design of intelligent Sia-conjugated CDs capable of targeting siglecs and other sialic acid binding proteins, paving the way for the development of clinically applicable CDs.



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Instrument and method development towards enhanced N-Glycan mass Spectrometry Imaging

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Abstract not provided

Highly sensitive spatial Glycomics at cellular resolution by in-situ derivatization and mass spectrometry imaging

Arun Everest-Dass[1]

1. Institute for Glycomics, Gold Coast, QUEENSLAND, Australia

Abstract not provided

SESSION 07

Glycolipid-peptide conjugate vaccines protect against liver-stage malaria

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Malaria is a highly prevalent parasitic disease, accounting for around 216 million infections and 445,000 deaths per annum. Although the anti-malarial vaccine, Mosquirix, was recently approved, this treatment is only modestly effective against clinical disease (achieving ~30% protection after 1 year and dropping to <10% protection after 4 years).

In the search for more effective treatments, we are developing glycolipid-peptide conjugate vaccines that expand a sub-population of non-circulating hepatic memory T cells - known as liver resident-memory T cells (T_{RM} cells) - which been shown to be 'front-line defenders' against various hepatotropic diseases including malaria.

In this paper, we report the design, synthesis and preclinical testing of glycolipid-peptide conjugate vaccines for the induction of T cell immunity in the liver and our progress towards developing new, efficacious vaccines for malaria.

Glycosylation-dependent protein quality control in malaria parasites enables transmission to mosquitoes and subsequent liver-stage infection

Sash Lopaticki[1], Robyn McConville[1, 2], Alan John[1, 2], Niall Geoghegan[1, 2], Shihab Deen Mohamed[1, 2], Lisa Verzier[1, 2], Ryan Steel[1, 2], Cindy Evelyn[1], Matthew O'Neill[1], Niccolay Madiedo Soler[1, 2], Nichollas Scott[3], Kelly Rogers[1, 2], Ethan Goddard-Borger[1, 2], Justin Boddey[1, 2]

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Malaria is a life-threatening infectious disease that kills over 620,000 people annually, predominantly young children. Malaria parasites are maintained between humans and mosquitoes in a complex lifecycle involving parasite motility, invasion and egress from host cells. The *Plasmodium* 'TRAP adhesin family' contain type I thrombospondin repeat domains and are expressed on the parasite surface at different lifecycle stages. The 'TRAP adhesin family' are required for parasite motility and host cell infection throughout the lifecycle. Despite common glycosylation being absent from malaria proteins, recently the presence of C- and O-glycosylation was discovered in the sporozoite stage of *Plasmodium falciparum* and *P. vivax*, which infect the liver in humans before establishing blood stage infection. We subsequently showed that O-glycosylation by POFUT2 is essential in *P. falciparum* for stabilising 'TRAP adhesin family' members that enable infection of mosquitoes and the liver. Here, we identify new substrates that are O- and C-glycosylated in *P. falciparum* and reveal the essential functions of a new glycosyltransferase, DPY19, responsible for tryptophan C-mannosylation in this human pathogen. Genetic disruption of *DPY19* attenuated *P. falciparum* gametocytes from egressing their human erythrocytes within the mosquito gut. This process was imaged to completion for the first time using lattice light-sheet microscopy, revealing critical defects in the absence of C-glycosylation. *DPY19*-deficiency also blocked *P. falciparum* ookinetes from invading the mosquito midgut, a critical step in malaria transmission. This work reveals that the most lethal malaria parasite of humans performs glycosylation in the endoplasmic reticulum for quality control of proteins required for transmission to mosquitoes and subsequent liver-stage infection. These are crucial steps in parasite dissemination through human and mosquito populations, providing potential avenues for transmission blocking interventions against this pernicious disease.

Treatment of Invasive Fungal Infections: discovery of new potential leads

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Opportunistic fungal pathogens, such as *Aspergillus, Candida,* and *Cryptococcus,* are leading causes of Invasive Fungal Infections (IFI). The spores of these fungal species are present in day to day living environments and are harmless to most people. However, to immunocompromised patients, it can cause severe life-threatening systemic infections such as Invasive Aspergillosis (IA). Immunocompromised patients include patients with HIV, diabetes, cancer, or an autoimmune condition. With the COVID-19 global pandemic resulting in an increase of hospitalisations, there has been a steep escalation in case numbers of COVID-19 associated IFI resulting from the increase in number of immunocompromised patients. Treatment of IFI however, has been complicated with the increase of drug resistance and the lack of new antifungals development. Using our innovative and proven drug discovery pipeline, we aim to tackle this increasingly serious threat to public health all around the world. Specific targets of the invading fungal cells have been identified which are absent in humans but play a critical role in the fungal virulence, such as the Galactomannan biosynthesis. This project has discovered three potential lead compounds which target a fundamental enzyme found in fungi, UDP-Galactopyranose Mutase (UGM). By targeting this enzyme, galactofuranose production is inhibited which impacts the Galactomannan biosynthesis, leading to fungal growth defects, altered virulence and subsequently fungal death. Using the innovative approach, the lead compounds have been analysed *in-silico*, and *in-vitro* with further work being conducted which will lead into *in-vivo* and *ex-vivo* studies.

A 'Genomics-Structure' approach to understanding polysaccharide biosynthesis in a critical priority bacterial pathogen

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2. University of Sydney, Sydney

The bacterial cell surface is an exquisitely diverse landscape comprising a variety of glycan and polysaccharide structures. For *Acinetobacter baumannii,* a bacterial species for which there are few effective antibiotic treatments remaining, the polysaccharide capsule (CPS) is a major virulence factor and target for new therapies. Individual strains produce just one of the large number of CPS types that is largely determined by which combination of genes are present at the chromosomal K locus (KL) for CPS biosynthesis. By assessing the CPS structure in light of the genome in the same strain, encoded proteins can be assigned to specific roles in the biosynthesis pathway revealing enzyme specificities without the need for time-consuming biochemistry. These assignments are strengthened as more CPS structures with corresponding genomes become available. Here, this 'genomics-structure' approach was used to characterise the CPS produced by an *A. baumannii* clinical isolate carrying the KL127 locus. Using NMR and chemical approaches, the CPS was found to be a polymer of pentasaccharide units. Correlation of this structure to KL127 revealed that the linkage between units was not the linkage expected to be formed by the encoded polymerase, indicating the presence of a polymerase gene elsewhere in the genome. A tailored bioinformatics approach enabled the finding of a candidate gene outside the K locus in a prophage region, and the additional finding that a strain with KL127 but not the prophage produced a structure with the expected linkage confirmed the role of the prophage gene in CPS biosynthesis. As structural changes can affect the specificity of antibodies and phage, this case study demonstrates that a 'genomics-structure' approach is a valuable workflow for detecting cases in which genes outside the K locus alter the CPS structure, thus providing novel insights into CPS biosynthesis and evolution for this species.

Identifying pathways of reserve carbohydrate biosynthesis in parasitic protozoa essential for virulence

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2. Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

Parasitic protists comprise an extremely diverse group of single celled eukaryotes that chronically or acutely infect more than 1/3 of the world's population and are responsible for diseases such as malaria, toxoplasmosis and leishmaniasis. Each of these pathogens target different host cells and tissue niches and have evolved markedly different growth strategies and capacity to establish long term chronic infections. Intriguingly, pathogens with the capacity to establish long term chronic infections, such as *Toxoplasma gondii* and *Leishmania* spp, universally synthesize intracellular carbohydrate reserves, which may allow these pathogens to switch to a slow growth, metabolically quiescent state and subsequently reactivate and cause acute infections. *T. gondii* synthesizes a plant like amylopectin (α1,4(α1-6 branched) glucan), while Leishmania have evolved a completely novel carbohydrate reserve, comprised of β1,2mannan chains, termed mannogen. We have identified novel proteins/enzymes involved in the synthesis of *T. gondii* amylopectin and *Leishmania* mannogen, including a repurposed trehalose-phosphate synthase and a novel family of GDP-Man:mannosyltransferases with dual mannogen phosphorylase activity (MTPs), respectively. Delineation of the function of these proteins demonstrates that reserve carbohydrate biosynthesis and turnover has a key role in regulating parasite central carbon metabolism and the capacity of these pathogens to persist long term within their mammalian host.

SESSION 08

A harmonised compositional and structural glycomics platform for the masses

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Protein glycosylation is the most frequent and varied type of co- and post-translational modification seen in eukaryotic cells. Identification of the glycans that modify individual proteins or protein mixtures (*e.g.* cell lysates and plasma) is valuable for hypothesis generation regarding the roles these glycans play in cellular physiology.

Differing glycan formats and analysis methods challenge reproducibility. Compositional analysis (*e.g.* MALDI) profiles glycans by their mass, whereas structural analysis (*e.g.* porous graphitised carbon{PGC}-LC-MS) separates isomers before detection, allowing structures to be selectively measured. Here, we describe two ESI methods to analyze released, native glycans with compositional or structural resolution, respectively. The first, a flow injection analysis MS method at 3 minutes per sample, and the second, a PGC-LC-MS method. Both methods analyse non-reduced glycans to simplify sample preparation, and avoid labile modification loss from alkaline conditions.

The compositional method was verified with a pilot treatment of ExpiCHO cells with Castanospermine, a known glucosidase inhibitor, showing accumulation of glucosylated *N*-glycans. As these results were consistent with expectations, a time-course study was performed with Kifunensin, an alpha 1,2 mannosidase, and Swainsonine, an alpha 1,3/6 mannosidase inhibitor. Similar to the Castanospermine treatment, inhibition was successful with an accumulation of glycans upstream from their respective inhibited biosynthetic enzymes.

Compositional analysis often struggles to connect detected glycans and their respective biosynthetic enzymes due to unresolved structural features. Using a set of model glycoproteins, we analyzed the glycans by our compositional method, then subsequently by PGC-LC-MS. LC optimization was performed to prevent anomeric peak splitting while maintaining glycan isomer separation. Diagnostic product ions from the structural analysis were translated to the MS2 spectra of the compositional data, enabling limited structural information to be extracted from previously composition-only data. With this harmonized platform, throughput can be tailored to the question instead of the reverse.

Multi-Glycomics: sequential release of the heterogeneous glycome from a single sample

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The complete glycome is structurally complex and diverse, composed of not only N- and O-glycans. Glycosaminoglycans (GAGs), glycosphingolipids (GSLs) and other distinct glycan features such as polysialic acids (PSA) and non-mucin type O-glycans (O-man, O-xyl) are also functionally significant. Various methods are used to analyse these different components of the glycome, but they require pre-fractionated/partitioned samples to target each glycan class individually. With this siloed approach, the capacity to determine the contribution of the total glycome to the biological function of interest is lost.

To address this need for a knowledge of the relationship between the different glycan components of a biological system, we have developed a sequential release workflow of analysis of multiple conjugated glycan classes (PSA, GAGs, GSLs, N-glycans O-glycans) from the same tissue lysate.

With this sequential glycan release approach, using mouse brain and heart tissue lysates as examples, five glycan classes were characterised from a single sample using enzymatic or chemical release of

the different glycan structures from a single sample spot dried onto a PVDF membrane. The released glycan classes were then analysed by HPLC and/or MS techniques. Interestingly, by comparison with the release of individual glycan classes from parallel samples, the removal of long negatively charged chains enhanced the yields of subsequent released glycans. Furthermore, the usually difficult to identify GAG cores, became visible in the O-glycan release.

Our sequential glycan release workflow thus not only improves the depth, but also the coverage, of the glycome. Importantly, as all the glycan classes were released from the same sample, relative quantitation of the different glycan structures to each other becomes possible. This coverage of the total heterogeneous glycome from only 40 micrograms of crude protein lysate makes this workflow a valuable approach to the deep characterisation of the complex glycome of cells and tissues.

Glycoprofiling by lectin-based glycoprotein microarrays

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Aberrant glycosylation is a hallmark of many diseases and physiological changes. Various bioanalytical methods can be used for its detection. We are focused on affinity techniques based on glycan binding molecules. Typically, lectins are used as biorecognition elements, which are proteins recognizing glycan structures enabling glycoprofiling of proteins, cells, and tissues. Our group develops lectin-based glycoprotein microarray assays in the reverse-phase format, the technique defined as a high-throughput approach enabling simultaneous and rapid analysis of a large cohort of samples by a set of lectins without the need for the separation of glycans from proteins. The microarray biochip is prepared by spotting of tens to hundreds of samples on the microarray substrate which is then allowed to interact with a panel of lectins. This method provides effective glycoprofiling of samples and screening/analysis of glycan biomarkers having huge applications in biomedicine, biology, and biotechnology. We have applied our microarray platform for the study of glycan changes in a number of various cases, e.g. cancer, gestational diabetes mellitus, congenital disorder of glycosylation (CDG), attention-deficit hyperactivity disorder (ADHD), age-related glycosylation changes, or glycostructure of therapeutic proteins.

Acknowledgement: APVV-20-0243, COST CA18132, APVV SK-SRB-21-0046. This publication is the result of the project implementation Centre for Advanced Therapies for Chronic Inflammatory Diseases of the Musculoskeletal System, ITMS2014+: 313011W410 supported by the Operational Programme Integrated Infrastructure funded by the European Regional Development Fund.

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A pain-causing and paralytic ant venom glycopeptide

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Venoms from animals and plants are a rich source of bioactive peptides. Post-translational modifications are key to the function of many venom peptides. I will present our discovery and characterization of an *O*-linked glycopeptide (Mg7a) which is a major component of the venom of the Australian giant red bull ant *Myrmecia gulosa*. Electron transfer dissociation and higher-energy collisional dissociation tandem mass spectrometry were used to localize three α-N-acetylgalactosaminyl residues (α-GalNAc) present on the 63-residue peptide. To allow for functional studies, we synthesized the full-length glycosylated peptide via solid-phase peptide synthesis, combined with diselenide–selenoester ligation-deselenization chemistry. We show that Mg7a is paralytic and lethal to insects, and

triggers pain behavior and inflammation in mammals, which it achieves through a membrane-targeting mode of action. Deglycosylation of Mg7a rendered it insoluble in aqueous solution, suggesting a key solubilizing role of the *O*-glycans.

SESSION 09

MAGMap: Liquid Chromatography Ion Mobility Mass spectrometry (LC-IM-MS) software for characterization of released glycans

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A significant burden in bio-manufacturing is the characterisation of glycans on viral vectors, vaccines, or biomolecules. Their characterization is time-consuming, requiring skilled manpower and expensive instruments. This results in slower and costly bioprocess development. To address the need for customized software to handle multi-attribute data arising from liquid chromatography ion mobility mass spectrometry (LC-IM-MS), we have developed the Multi-attribute Glycan Map (MAGMap) software presented here. **MAGMap is an automated glycan characterization software designed to process and visualize multi-attribute glycan data**. The software can process raw data from the mzML data standard and extract multiple attributes such as Glucose Units (GU) from the LC, mass to charge ratio (m/z) from the MS and collision cross section values from the IM. Using the extracted multi-attribute information, the software can automatically identify glycans using database matching and data visualization approach more accurately than LC-MS alone.

Evaluating confidence score relationships across glycopeptide search engines via a novel glycan database and glycopeptide result converter

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Multiple glycoproteomics search engines are available, including Byonic, pGlyco3, Metamorpheus, however, their utility is limited by the lack of a harmonized input/output format. Thus, search results cannot be directly compared, and users are relegated to design approaches to correlate resultant data. Moreover, search engines apply different algorithms to calculate output “confidence scores”. Here, we present a novel application to convert the output of several commonly used glycan database and search engine formats. We applied our conversion program to human serum samples analyzed using pGlyco3, Byonic, and MetaMorpheus and report confidence score dynamics for the results.

A *N*-glycan database for the three search engines was built and identical parameters were run to yield glycopeptide results. We used our converter on pGlyco3 input files for 154 *N*-glycan compositions from Byonic’s human plasma database. Glycopeptide identification was achieved using pGlyco3, Byonic, MetaMorpheus search engines with the same parameters. Our glycopeptide result converter was applied to extract specific information including: MS2 Scan number, peptide sequence, glycoform, retention time, and confidence score output for each search engine. Confidence score comparisons were compared by reverse tracing of the scan numbers for individual experiments.

For samples analyzed across five FAIMS collisional voltages (35V, 40V, 45V, 50V, 55V), pGlyco3 identified 3,038-6,801 glycopeptide-containing spectra (GCS), Byonic identified 4,660-9,737 GCS, and MetaMorpheus identified 3,140-6,818 GCS. Since our converter retrieves MS2 scan numbers and confidence scores, conversions between confidence scores for different search engines could be analyzed based on the same MS2 scan. Without any confidence score filters, 30.1% (3,266 of 10,868 PSMs) of glycopeptide containing spectra reported a shared space at FAIMS CV 45V by the pGlyco3, Byonic, and MetaMorpheus search engines.

In conclusion, our glycopeptide converter enabled a comparison of the output results from pGlyco3, Byonic, and MetaMorpheus while accounting for differences in the respective confidence scores.

Biocuration of Glycosylation Data

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GlyConnect (https://glyconnect.expasy.org/) is part of a suite of resources hosted by the Swiss Institute of Bioinformatics. The resources include tools to interrogate curated glycosylation data stored in GlyConnect. The central theme is the glycan composition/structure. These structures are identified by a number of analytical techniques and have varying levels of detail. They are represented in the standard SNFG nomenclature [1] which allows efficient and rapid understanding of experimental glycan structures.

As biocurators of this type of data, we have identified a number of bottlenecks and potential solutions. The main one is the time-consuming re-interpretation of glycan structures. Published glycan structure data is represented in many different ways; from lists of potential compositions derived from proprietary databases to annotated tables containing m/z, charge state, abundance and possibly SNFG cartoons. There are two consequences of the reliance on images: bioinformatics tools cannot interrogate them and, second, while informative, they may not always reflect the intended structure e.g. in a tri-antennary N-Glycan, what is the evidence for the antennae position?

These consequences lead to time-consuming “re-annotation” of structures, and trawling of papers for evidence. The futility of the curation task is worsened by the fact that cartoons found in papers are very likely to have been created with tools that have the capability to output the structure in a computer-readable format. Ideally, a structure identifier (GlyTouCan, https://glytoucan.org/) should be provided as it links directly to multiple formats.

We have created several tools, including a simple standalone tool that interrogates GlyConnect and then GlyTouCan for the presence of structures and returns IDs from both of these resources if present. Input is a GWS string which is the default output of GlycoWorkBench/GlycanBuilder [2]. Adopting new habits in data submission with inclusion of structure formats will positively impact the reporting and biocuration of experimental results.

1. [1] Neelamegham S, Aoki-Kinoshita K, Bolton E, Frank M, Lisacek F, Lütteke T, O'Boyle N, Packer NH, Stanley P, Toukach P, Varki A, Woods RJ; SNFG Discussion Group. Updates to the Symbol Nomenclature for Glycans guidelines. Glycobiology. 2019 Aug 20;29(9):620-624. doi: 10.1093/glycob/cwz045. PMID: 31184695; PMCID: PMC7335484.
2. [2] Damerell D, Ceroni A, Maass K, Ranzinger R, Dell A, Haslam SM. Annotation of glycomics MS and MS/MS spectra using the GlycoWorkbench software tool. Methods Mol Biol. 2015;1273:3-15. doi: 10.1007/978-1-4939-2343-4_1. PMID: 25753699.

Using inference on Semantic Web data to enrich the data in GlyCosmos

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The GlyCosmos Portal [1] has been developed using Semantic Web technologies, meaning that the data stored in GlyCosmos maintains the semantics, or the relationships, between data records. For example, to represent a glycoprotein X with a glycosylation site Y occupied by a particular glycan Z identified by a GlyTouCan ID G00000, we can represent this information by storing the following triples: (a) X has_glycosylation_site Y (b) Y is_occupied_by Z (c) Z has_identifier “G00000”. The predicates (e.g., “has_glycosylation_site”) are predefined in an ontology which encapsulates its meaning; X, Y and Z are identified by a universal resource locator, or URI, as if it was an address on the Internet. Thus, if

another database also contains Semantic Web data regarding protein X, it could be identified uniquely by the URI for X, without needing to perform any mapping or data exchange.

Having reached version 3.0 in August, 2022, we attempted to perform inference on the Semantic Web data in GlyCosmos. As a simple proof-of-concept, we focused on the organisms that have been accumulated across glycans, glycoproteins, pathways, diseases, etc. in GlyCosmos. As of version 3.0., there were species whose distinct strains were annotated to contain different glycans, but taxonomies that were at higher levels to that species (such as genus or kingdom) were not annotated with the same data, despite the fact that logically, they should contain all the annotations that have been applied to the species within its hierarchy. For example, the species *Aspergillus oryzae* has 16 glycans, 5 glycoproteins, and 1 lectin entry. However, the genus *Aspergillus* was annotated with only 10 glycans, whereas it should also contain the glycans, glycoproteins, etc. for all of the species under the *Aspergillus* genus.

Here, we used the semantics in the GlyCosmos database to perform inferences on these taxonomic annotations. First, we obtained the whole taxonomic hierarchy from NCBI and stored it in our datastore as triples. Then, we formulated inference rules to automatically obtain the higher taxonomies for any particular taxon. As a result, we were easily able to formulate queries that would automatically annotate the information from the lower taxons to the higher taxons, thus returning information that was semantically accurate. Note that we did not have to generate any new data to specifically indicate that the data associated with a lower taxon was also associated with the higher. Thus, we were able to illustrate the power of Semantic Web technologies, and we plan on incorporating these techniques into GlyCosmos in future releases.

1. I. Yamada et al., “The GlyCosmos Portal: a unified and comprehensive web resource for the glycosciences,” Nat. Methods, vol. 17, no. July, pp. 649–650, 2020, doi: 10.1038/s41592-020-0879-8.

SESSION 10

The application of synthetic biology to the production of O-glycosylated proteins in *E. coli*

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Mucin-type O-glycosylation is initiated by the addition of α-linked GalNAc to serine or threonine residues by enzymes in the GT27 family of glycosyltransferases. Unlike their N-linked counterparts, O-GalNAc glycans do not have a precise consensus amino acid sequence (sequon) where the transfer takes place. Some general considerations have emerged based on natural sites of glycosylation as well as extensive synthetic peptide modification work, which we have exploited to engineer bacterial strains for the production of sialylated Core 1 glycan structures on human therapeutic proteins. We have used a two plasmid system combining genes from bacterial, and mammalian sources to permit the production of a variety of proteins with O-glcyans of the sialylated core 1 type. We will present a summary of progress to date in developing this system toward an industrially useful expression system for therapeutic cytokines.

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Glycopeptide reporters of early-stage colorectal cancer

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Australia has the highest incidence rate of colorectal cancer (CRC) worldwide. Although novel treatments for CRC have emerged, the cure rates and long-term survival remain dishearteningly low. Non-invasive biomarkers for the early detection of CRC are urgently needed to guide clinical decision-making and ultimately increase the patient survival chances. To this end, we here present a paired discovery and targeted glycoproteomics workflow that measures site-specific *N*-glycosylation changes of key glycoproteins directly from non-depleted plasma of CRC patients spanning four disease stages (Dukes I-IV, n = 7/stage) and age/gender-matched healthy donors (n = 8). For the discovery-based approach, TMT labelling, high-pH fractionation and HILIC enrichment of the *N*-glycopeptides were employed prior to LC-MS/MS-based identification and quantitation of ~2,000 intact *N*-glycopeptides covering 76 *N*-glycoproteins. While we generally showed relatively stable plasma protein expression across the CRC stages and controls, the site-specific *N*-glycosylation patterns of several highly abundant plasma glycoproteins, including haptoglobin, immunoglobulins and complement C3 were significantly altered between specific CRC stages relatively to the healthy controls. To validate the findings with a clinically-relevant assay featuring minimum sample handling, we developed a label-free targeted glycoproteomics method employing scheduled parallel reaction monitoring (PRM) on a quadrupole-orbitrap mass spectrometer for the sensitive detection and quantification of 16 *N*-glycopeptides from five plasma glycoproteins including haptoglobin, IgG and complement C3. The PRM method provided accurate AUC-based *N*-glycopeptide quantification by the targeted detection of multiple oxonium ions (*m/z* 138, 204, 366) and peptide-specific Y1 and Y2 fragment ions. When adjusted for the protein level variations, the PRM-based glycopeptide quantification namely recapitulated the stage-specific changes observed in the discovery-based glycoproteomics approach but at considerably higher sensitivity and speed. In summary, we have developed a promising clinically-relevant quantitative method for the discovery and validation of protein-specific glycosignatures in plasma featuring a translational potential for the early detection of CRC.

Engineering the lectin activity of a bacterial toxin to create a new tool for cancer diagnosis and monitoring

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Aberrant glycosylation is one of the hallmarks of cancer cells. Glycans terminating with the sialic acid, N-glycolylneuraminic acid (Neu5Gc) are not expressed at significant levels on healthy human tissues. Nevertheless, Neu5Gc-containing glycoconjugates have been consistently found in human tumor tissues, cells and secretions, and have been investigated as tumor biomarkers for decades. Until recently, sufficiently specific and practical tools for the detection of Neu5Gc in a clinically relevant biological sample, such as serum, have been lacking. Certain Shiga toxigenic Escherichia coli (STEC) strains produce subtilase cytotoxin (SubAB) that preferentially recognizes α2-3 linked Neu5Gc via its pentameric B-subunit SubB. We used structure aided design to engineer the SubB lectin to ablate the recognition of "normal" Neu5Ac and enhance recognition of cancer-related Neu5Gc[1]. Using this novel, Neu5Gc-specific lectin, termed SubB2M, we have detected elevated Neu5Gc glycoconjugates in serum from ovarian cancer patients at all stages of disease compared to healthy controls, in a highly sensitive surface plasmon resonance (SPR)-based assay[2]. A similar study using cancer-free and Stage I-IV breast cancer patient serum samples revealed that Neu5Gc serum biomarker levels can discriminate breast cancer patients from cancer-free individuals[3]. Moreover, analysis of serum collected prospectively, post-diagnosis, from breast cancer patients at high risk for disease recurrence showed a trend for a decrease in Neu5Gc levels immediately following treatment for those in remission. Neu5Gc serum biomarkers are a promising new opportunity for early detection and disease monitoring of ovarian and breast cancer and may have utility for screening and monitoring of other types of cancer.

1. Day et al, 2017, Sci Rep, 7, 1495
2. Shewell et al, 2018, BBRC, 507, 173-177. Shewell et al, 2022, BMC Cancer, 22, 334.

Glycosaminoglycans that enhance mesenchymal stem cell banking

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A loss in therapeutic potency of allogeneic banks of human bone marrow-derived mesenchymal stem cells (MSC) following serial passaging is a major bottleneck for clinical and commercial applications. Prospectively screening of bone marrow donors for genomic biomarkers that predict the ability of isolated MSC populations to be expanded without a loss of potency offers a unique opportunity to create banks of high-quality MSCs. Additional use of media formulations containing growth-factor potentiating glycosaminoglycans (GAGs) are being developed to further retain MSCs potency during *ex vivo* expansion. Such glycosaminoglycans bind and stabilise important MSC mitogens (like FGF2) and sustain proliferative signals in MSCs without compromising their capacity for multilineage differentiation. Notably, MSCs cultured in GAG-enriched media formulations are highly effective at regenerating osteochondral defects in rodent and porcine models. This seminar will describe the discovery science and how integrating these strategies into a bioprocess for the manufacturing and banking of MSCs is critical to the translation of this GAG-based program.

SPEED TALKS & POSTERS

#01 - Spatial distribution, protein carriers and cellular origins of paucimannosidic glycosignatures in colorectal cancer tissue

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Colorectal cancer (CRC) is a prevalent and lethal disease featuring a complex tumour microenvironment (TME). The CRC TME is known to harbour cancerous intestinal epithelial cells, various immune cell types, stroma and extracellular matrix engaging in glycan-mediated cell-cell communication that facilitates key tumourigenic processes. Building on our recent observation that paucimannosidic N-glycans are elevated in CRC tissues [1], this study aims to spatially profile paucimannosidic glyco-epitopes and identify their protein carriers and cellular origin(s) in the CRC TME. Two complementary approaches were employed to investigate a valuable collection of FFPE slides of paired tumour and normal adjacent colon tissues from CRC patients- i) a qualitative immuno- and lectin histochemical staining approach targeting paucimannosidic glycoepitopes and cell-specific markers and ii) quantitative LC-MS/MS-based glycomics and glycoproteomics. Histochemistry revealed that paucimannosidic glycoepitopes are largely confined to the stroma of the CRC TME, and, interestingly, that these co-localise with M2 macrophages (CD163) as opposed to intestinal epithelial cells (EpCAM). The PGC-LC-MS/MS glycomics data mapped the fine structures of several paucimannosidic glycans and, as expected, demonstrated that paucimannose levels were significantly elevated in CRC tumour tissues relative to normal adjacent tissues. Finally, the glycoproteomic data revealed the identity of the paucimannose-carrying proteins, and, indirectly, their cellular origins by mapping data back to the Human Protein Atlas. As a prominent example, myeloperoxidase, a glycoprotein expressed by M2 macrophages, was found to be dramatically elevated in CRC tumour tissues relative to normal adjacent tissues and was decorated abundantly with paucimannosylation at multiple sites. Collectively, our data suggest that the paucimannosidic signatures in the CRC TME are of innate immune origins. The study expands our understanding of the spatial distribution and cellular origins of overlooked glycoepitopes in the CRC TME, fundamental knowledge important to better delineate CRC onset and progression and develop new therapeutics against the disease.

1. [1] Chatterjee S, Lee LY, Kawahara R, Abrahams JL, Adamczyk B, Anugraham M, Ashwood C, Sumer-Bayraktar Z, Briggs MT, Chik JHL, Everest-Dass A, Förster S, Hinneburg H, Leite KRM, Loke I, Möginger U, Moh ESX, Nakano M, Recuero S, Sethi MK, Srougi M, Stavenhagen K, Venkatakrishnan V, Wongtrakul-Kish K, Diestel S, Hoffmann P, Karlsson NG, Kolarich D, Molloy MP, Muders MH, Oehler MK, Packer NH, Palmisano G, Thaysen-Andersen M. Protein Paucimannosylation Is an Enriched N-Glycosylation Signature of Human Cancers. Proteomics. 2019 Nov;19(21-22):e1900010. doi: 10.1002/pmic.201900010. Epub 2019 Oct 16. PMID: 31419058.

#02 - Utilisation of Cyclic Ion Mobility with multiple pass acquisition for the analysis of glycopeptides and glycoforms associated with SARS-CoV-2

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Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to be responsible for the large-scale epidemic globally. The SARS-CoV-2 S

protein is highly conserved and involved in multiple processes, including receptor recognition and viral attachment. The viral S protein is modified by glycosylation which may be implicated in immune evasion from the host immune system by shielding the protein surface from detection by antibodies, affecting the ability of the host to mount an effective adaptive immune response. It has therefore become an important target for vaccine research. Here, we demonstrate the utility of Cyclic IMS (cIMS) for in-depth glycopeptide characterisation using the multi-pass feature to separate co-eluting glycoforms related to the SARS-CoV-2 S1 protein.

The SARS-CoV-2 S1 protein, which contains a His Tag (S1N-C52H3) and is expressed from human 293 (HEK293) cells, was used as an example protein to illustrate the benefits of the multi-pass functionality. The sample was reduced, alkylated and digested with trypsin prior to LC-MS analysis. The corresponding peptides were separated using one-dimensional liquid chromatographic (LC) configured as trap and elute and utilising a 75 micron analytical column. 20ng of digest was loaded and separated over a 30min gradient. The LC was coupled to an IMS oa-QTof MS and data were obtained using an ion mobility enabled DIA method, HDMSE and targeted HDMSMS. Data were processed using a variety of informatic tools in order to provide annotated glycopeptide identification.

Initial assessment of the digest was conducted using HDMSE, which comprised of a single pass of the cyclic device. Based on the initial, single pass data and observation of typical oxonium ions within the fragment ion spectra, glycopeptides at m/z 969.08 (3+) and 1262.9 (3+) were selected as candidates for further investigation using quadrupole isolation and multi-pass ion mobility. In order to allow multi-pass acquisitions, the cIMS settings were configured with mobility separate times derived from the instrument control software pass calculator and by measuring the ion behaviours for 1 and 2 passes. A total of five passes of the cyclic device was sufficient to show the emergence of multiple glycoforms for the ion at m/z 1262.9 and it was found that the fragment spectra corresponding to this species had the characteristic sialic acid linkage (m/z 657.2). Further experiments, which consisted of up to ten passes of the cyclic device, did not result in additional information. Interpretation of the glycopeptide sequences was conducted using the GlycReSoft software package, in addition to manual interpretation as further validation.

#03 - Glycobiology in 3D – informatics, quality control, model building and molecular dynamics

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Carbohydrates are involved in many biologically important molecular recognition processes, which ultimately trigger a specific biological function. Since the binding strength (affinity) of a molecular complex is determined by the complimentary of the interacting molecular surfaces, the knowledge of the three-dimensional (3D) structure of molecules is required in order to fully understand biological function on the molecular level. The Protein Data Bank (PDB) (www.wwpdb.org) currently contains almost 200000 experimentally determined 3D structural models of biomolecules and their assemblies, including proteins, nucleic acids and carbohydrates. About 7% of the PDB entries do contain at least one monosaccharide, which makes the PDB a valuable resource for data-mining in structural glycobiology [1]. However, it should be noted that glycans in PDB entries are typically incomplete and the generation of full 3D structural models of a glycoprotein requires the application of molecular modeling methods. Additionally, a significant number of carbohydrate components in the PDB do have unrealistic high-energy conformations. Consequently, it is important to have software methods available that allow for carbohydrate detection, model building and quality checks in a given 3D structure. Here I do present *Conformational Analysis Tools (CAT),* a powerful software developed over more than 20 years that supports automated workflows to build 3D models of glycoproteins, protein-carbohydrate complexes and polysaccharides with high quality and to analyse their dynamics and molecular interactions. CAT has recently been used for example to analyze the structure, dynamics, receptor binding, and antibody binding of the fully glycosylated full-length SARS-CoV-2 spike protein in a viral membrane and to build a model of the fully glycosylated Ebola glycoprotein (https://orcid.org/0000-0002-1006-6746).

1. T. Lütteke, M. Frank, C.-W. von der Lieth (2004) Data mining the protein data bank: automatic detection and assignment of carbohydrate structures. Carbohydr Res 339:1015–1020

#04 - A New Synthetic Method for Asymmetrically-Substituted Trehalose Derivatives

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This project aims to develop a new strategy to asymmetrically-substituted trehalose derivatives via a borinic acid-catalyzed 1,2-*cis*-stereoselective glycosylation,[1] which can be applied to the synthesis of mycobacterial lipooligosaccharides (LOSs).[2]

Many glycans in the mycobacterial cell wall (MCW) help these organisms escape detection from the host immune system. Despite their roles in influencing the progression of mycobacterial diseases, we have a poor molecular-level understanding of how MCW glycans exert their effects. Among the many carbohydrate components in MCW, mycobacterial LOSs are the focus of this research, as they display tremendous structural diversity and there is a lack of efficient methods for their synthesis.

All LOSs share a common trehalose core which is an α,α-1,1-disaccharide composed of two glucopyranose residues. Although there have been reports for synthesizing a range of trehalose derivatives, these methods are not suitable for molecules like mycobacterial LOSs, which have complex acyl and glycosyl substitution patterns. A general approach to asymmetrically substituted trehalose derivatives involves the preparation of two different glucose derivatives and a glycosylation reaction for coupling the two residues together. However, constructing the 1,1-glycosidic linkage in a stereoselective manner at both anomeric centres is difficult. Four possible stereoisomers (αα, αβ, βα and ββ) are possible, which can cause low yields and difficulties in purification. In this research, we have applied borinic acid-catalyzed 1,2-*cis*-glycosylation methodology to the synthesis of trehalose units, using 1,2-α epoxy glucoside donors and hemiacetals as acceptors. The set of trehalose building blocks thus produced will be useful in synthesizing mycobacterial LOSs with different acylation patterns; for example, this method will be used for the synthesis of Mycobacterium gastri LOS I–IV. After synthesizing a range of LOSs, a plate-bound microarray of mycobacterial LOSs will be produced and utilized as a probe to unravel their biological activities.

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2. Angala, S. K.; Belardinelli, J. M.; Huc-Claustre, E.; Wheat, W. H.; Jackson, M. Crit. Rev. Biochem. Mol. Biol. 2014, 49, 361–399.

#05 - Examining the salivary N-glycome during experimental *Streptococcus pyogenes* infection

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Streptococcus pyogenes is a significant human pathogen is responsible for a diverse range of tissue infections. Its primary niche is the oropharynx which is rich in glycans, some of which are implicated in the innate immune response. Using a time-course approach, we are characterising the dynamic

oropharyngeal glycoprofile in saliva during experimental human pharyngitis with *emm*75 *S. pyogenes* (CHIVAS-M75 study).

Baseline, acute, and convalescent saliva samples were collected from healthy adult participants challenged with emm75 *S. pyogenes.* Qualitative and quantitative analysis of N-glycans extracted from saliva was performed by porous graphitised carbon liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (PGC-LC-ESI-MS/MS; LC-MS).

Of 145 N-glycans detected in saliva, 108 have been structurally solved or predicted from elution profiles and MS2 analyses. The majority (60%) are complex N-glycans. Preliminary time-course analyses from one patient reveal changes in the glycoprofile 48 hours post-infection. The major change is an increase in N-glycans bearing neuraminic (sialic) acid, which has a well-recognised role in the innate immune response. This effect is mediated primarily by complex, doubly-sialylated structures.

The host glycome is an important research target for efforts to understand host-pathogen interactions in streptococcal pharyngitis, and these findings will be validated in a larger cohort of human challenge trial participants. The observed sialic acid response suggests it may have an important role in triggering innate immune responses to *S. pyogenes* mucosal infections.

#06 - Hyper-truncated *N*-glycosylation of neutrophil azurocidin enables binding to *P. aeruginosa* PA-IIL (LecB)

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Azurocidin is a key microbicidal *N*-linked glycoprotein expressed by granulated neutrophils within our innate immune system, but the site-specific structure and biological roles of the *N*-glycans decorating azurocidin remain unknown. To this end, we have performed a deep structure-function characterisation of the *N*-glycosylation carried by azurocidin isolated from human neutrophils. Using PGC-LC-MS/MS glycomics, we firstly show that a paucimannosidic *N*-glycan ($Man_2GlcNAc_2Fuc_1$, M2F) is a highly abundant (~55%) structure of azurocidin. Glycopeptide-focused LC-MS/MS analysis supported the glycomics data and revealed that two of three azurocidin *N*-glycosylation sites (Asn126 and Asn140) are prominently decorated by an unusual chitobiose core *N*-glycan (i.e. $GlcNAc_1Asn$) while a third site (Asn171) is predominantly occupied by M2F. Importantly, LC-MS/MS-based top-down analyses recapitulated the bottom-up data and revealed a heterogeneous glycoform profile of intact azurocidin dominated by hyper-truncated *N*-glycans rich in unusual Man, GlcNAc and Fuc glycoepitopes and an absence of other post-translational modifications. We therefore probed the binding potential of the fucose- and mannose-binding *Pseudomonas aeruginosa* lectin, PA-IIL (LecB), to azurocidin. Interestingly, recombinant tetrameric LecB displayed considerable affinity to azurocidin using a simple in-solution binding assay while no LecB binding was observed to oligomannosidic and sialylated bovine RNAse B and fetuin, respectively. Notably, both native MS and mass photometry provided further evidence supporting a LecB-azurocidin complex formation, while 3D structural modelling unveiled that two strategically-positioned *N*-glycans on the surface of azurocidin at Asn126 and Asn140 are compatible with a potential bivalent binding mechanisms to the glycan-binding domains of tetrameric LecB. In conclusion, we have demonstrated that neutrophil azurocidin exhibits a highly unusual and site-specific hyper-truncated *N*-glycophenotype that displays binding to LecB. The findings of this study open for new avenues to explore how unusual neutrophil *N*-glycans impact host-pathogen interactions in the human innate immune system.

#07 - Distinct N-glycan signatures of cancer and immune cells from ovarian cancer ascites

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The accumulation of ascites fluid in the peritoneal cavity is a hallmark in patients with epithelial ovarian cancer. Ascites has the potential to serve as a source of biomarkers to better characterise the disease and to study treatment response. In this study we sought to characterise the N-glycome of the two major cell types in ascites (cancer and immune cells) from patients with chemo-naïve high-grade serous ovarian cancer.

Flow cytometry was used to isolate CD45+ (immune) and CD45-/EpCAM+ (cancer) cells from three patients. As a comparison, a sample of total cells from the same ascites samples before cell sorting was included. Cell lysates were immobilised onto PVDF membrane for N-glycan release followed by analysis of reduced N-glycans by PGC-LC coupled with negative ion mode MSMS.

In total, 192 N-glycans were identified in whole ascites, and a qualitative comparison of structures between cancer and immune cells identified 26 N-glycans detected only in immune cells and 42 N-glycans detected only in cancer cells. In regards to glycan classes, a quantitative comparison (relative % abundance) showed that the cancer cell N-glycome displayed biosynthetically less processed structures with 52% contributed by oligomannose structures compared to 20% complex type structures. Immune cells showed slightly more complexity with 30% complex type structures and 44% oligomannose type. Excitingly, when the EpCAM+ and CD45+ population 'sub-glycomes' were compared to the profile of total ascites cells, we found 61 N-glycans only detected in the EpCAM and CD45 marker negative population suggesting that other ascites cell types may also harbour a distinct N-glycome.

These results indicate that N-glycosylation signatures are able to distinguish sub-populations of cells derived from ovarian cancer ascites. This work sets the basis to further explore the cell type-dependent glycosylation in patient material, ultimately, during disease progression (to identify possible new drug targets) and/or treatment response.

#08 - Comprehensive Glycoproteome Profiling of Resting and Thrombin-Activated Platelets

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Platelets play central roles in the vascular and immune systems, including in haemostasis, thrombosis, inflammation, host defence, and carcinogenesis. Tissue injury promptly activates resting platelets, triggering profound morphological changes and granule exocytosis, resulting in the release of granular proteins (releasate) that mediate injury-related response processes. Despite the documented importance of protein glycosylation in platelet biology, the platelet glycoproteome remains poorly defined. This study employs complementary glycomics and glycoproteomics approaches to comprehensively map the *N*-glycoproteome of the lysate and releasate of resting and thrombin-activated platelets. Primary platelets were isolated in their resting condition from blood of healthy donors. Platelets were left unstimulated (n=10) or were either partially (n=6) or fully (n=5) activated with α-thrombin, a potent platelet agonist. Thrombin-mediated platelet activation was confirmed using PAC-1- and CD62P-centric flow cytometry. The releasate fractions were then collected and separated from platelet cellular fractions (lysate). Firstly, the *N*-glycome of the platelet lysate and releasate was

quantitatively profiled using PGC-LC-MS/MS-based glycomics. Platelet lysates and releasates displayed profound *N*-glycome diversity rich in sialylated and core-fucosylated complex-type *N*-glycans across both resting and activated conditions. Excitingly, a thrombin dose-dependent elevation of sialylated and fucosylated complex-type *N*-glycans displaying a higher degree of branching and a higher global protein occupancy with a concomitant reduction in bisecting GlcNAcylation were observed in the releasate under activated conditions. The *N*-glycoproteomics data recapitulated and expanded on the glycomics findings by uncovering a total of 339 unique *N*-glycopeptides from 92 different source *N*-glycoproteins, the highest coverage to date, and suggested that platelets exhibit subcellular-specific *N*-glycosylation featuring prominent sialofucosylation in the α-granules, paucimannosylation in lysosomes and, surprisingly, oligomannosylation on the platelet surface. Taken together, this study provides the hitherto most detailed view into the *N*-glycoproteome of resting and activated platelets, forming a valuable resource to further explore the fascinating platelet glycobiology in human health and disease.

#09 - MicroGlycoDB: a prototype database of microbial glycan-related information

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Since glycans play important roles in living organisms, databases have been developed to organize glycan information, but it has mainly focused on mammalian organisms. Only a few glycan-related databases for microorganisms have been developed. Therefore, our laboratory has developed “MicroGlycoDB” a database of microbial glycans and their related genes, using Semantic Web technologies, in order to better understand biological processes such as enzymatic reactions in which microbial glycans are involved. This presentation describes the updates to the data in MicroGlycoDB over the past year. Previously, glycan and pathway data for *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Campylobacter jejuni*, *Cryptococcus neoformans*, and *Mycobacterium abscessus* were available. Since then, new data on *Mycobacterium tuberculosis* have been added. Data such as gene numbers and text representation of glycan structures were received from collaborators. UniProt IDs were obtained from gene numbers in UniProt, and resources from related external databases such as Rhea, ChEBI, KEGG and CAZy were collected. The collected data were converted to Resource Description Framework (RDF) format based on the GlycoRDF[1] glycan ontology. In addition, the glycan structures were illustrated in SNFG format using an editing tool provided by the Carbohydrate Structure Database (CSDB)[2]. The data was loaded into the RDF database, APIs to query it were created, and the user interface was designed to access these APIs. This data has also been integrated into GlyCosmos. As a result, *Mycobacterium tuberculosis* can now be selected from the top page. The *Mycobacterium tuberculosis* main page displays the glycosylation pathway for each glycan type, and tables of glycan-related information and gene-related information are displayed as well. In addition, a detailed page summarizing gene- and glycan-related information has been introduced for each individual gene and glycan, respectively. Currently, we are working a system that will allow users to semi-automatically input their data into MicroGlycoDB online.

1. [1] GlycoRDF https://academic.oup.com/bioinformatics/article/31/6/919/214750
2. [2] CSDB/SNFG structure editor http://csdb.glycoscience.ru/snfgedit/snfgedit.html

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#10 - Investigating the CASD1 enzyme as a novel therapeutic target against melanoma

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Despite the tremendous advancements in the clinical management of cutaneous melanoma since the introduction of targeted therapy and immunomodulation, melanoma remains the most lethal of all skin cancers. Queensland residents have a 1 in 38 chance of developing melanoma, one of the world's highest, placing a tremendous burden on individual lives and the Country's healthcare system[1]. Melanoma's mutational inter- and intra-tumour heterogeneity have so far hindered efforts towards developing effective and universal anti-melanoma therapeutics. However, findings that the expression of cell surface 9-*O*-acetylated GD3, which reduces a cells' ability to undergo apoptosis, is upregulated throughout the different stages of melanoma growth, has alluded to the identification of CASD1 as being a potential therapeutic option. CASD1 is responsible for the enzymatic addition of an acetyl group to the C-9 hydroxyl group (C9-OH) of the sialic acid glycerol side-chain, a process referred to as 9-*O*-acetylation[2]. The objective of this study is to identify and validate putative inhibitors of the CASD1 enzyme. To this end, a library of small-fragments and peptide-based inhibitors were analysed by Surface Plasmon Resonance (SPR) for potential CASD1 interactions. Identified hits were evaluated by means of both CASD1 activity inhibition assays, as well as *in-vitro* cytotoxicity assays using melanoma as our cancer model. Here we present our preliminary findings aimed in identifying lead compounds that could be further developed into CASD1 targeted therapeutics and block the action CASD1 that enables cancer cells to evade CASD1 mediated anti-apoptotic signalling. Excitingly, several putative CASD1 inhibitors were identified and validated as having both inhibitory and cytotoxic activity.

#11- Ganglioside GM3 interacts with basic amino acids just above the transmembrane domain of the insulin receptor

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Biochemical and cell biological experiments have shown that GM3, a cell membrane glycolipid, inhibits the function of insulin receptor (IR). However, due to the heterogeneity of the cell membrane, it has been difficult to analyze the interaction between GM3 and IR in detail. In this study, we synthesized a fluorescently labeled transmembrane peptide (NBD-IR-TM) and incorporated it into liposomes to construct a simplified model system for interaction analysis.

The localization of NBD-IR-TM in phase separated GUVs containing glycolipids was analyzed. The co-localization of NBD-IR-TM with GM3 was significantly increased compared to that of lactosylceramide (LacCer), a GM3 precursor without sialic acid. Furthermore, the fluidity of NBD-IR-TM in GUVs was significantly reduced by the addition of GM3; in MLVs, the association of NBD-IR-TM was inhibited by GM3, but not by LacCer. Finally, we analyzed the aggregation state of two model peptides with and without charge, and found that only the association of the positively charged peptide was inhibited by GM3. These results indicate that the basic amino acids and acidic sugars of each molecule are important for the interaction between transmembrane peptides and GM3, and that this electrostatic interaction also occurs between the insulin receptor and GM3.

#12 - Quantitative Analysis of Galectin-dependent Glycoprotein Dynamics in Synthetic Glycan Chemical Knockin Cells

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Glycans form interaction networks with various molecules on the cell surface to regulate the dynamics and signal transduction of membrane molecules. The temporal and spatial structural diversity of glycans makes it extremely difficult to correlate the glycan structures with the function of membrane molecules. Therefore, we developed a new system that uses HaloTag® technology to display synthetic homogenous glycans on the cell surface (synthetic glycan chemical knockin). In this study, we applied this system to analyze the dynamics of glycan-displaying proteins through the interaction of galectin-3 and various glycans.

Galectin-3 (Gal-3), which recognizes β-galactose-containing glycans, binds to glycoproteins and glycolipids on the cell surface (1) to form a network structure called galectin lattice. Although the galectin lattice has been thought to regulate membrane protein function by suppressing lateral diffusion and endocytosis of membrane proteins, it has been difficult to precisely examine the galectin lattice function depending on glycan structures. Thus, we used the fluorescence recovery after photobleaching (FRAP) and single-particle tracking (SPT) methods to measure the lateral diffusion rate of the protein having the homogeneous synthetic glycan. FRAP analysis showed that the lateral diffusion of membrane proteins, in particular, those with Gal-3 high-affinity glycan ligands such as Gal-GlcNAc repeats, was significantly suppressed by Gal-3. SPT analysis showed a clear glycan structure dependence of protein lateral diffusion suppression by Gal-3. Furthermore, the Gal-3 treatment divided the lateral diffusion of the target protein into two major components, depending on the inhibition degree. From these results, we concluded that the galectin lattice not only reduced the entire cell membrane fluidity, but also suppressed the diffusion of target proteins by trapping them via glycans recognized by Gal-3.

In conclusion, we have succeeded in analyzing the function of glycan-lectin interactions for each glycan structure by using a synthetic glycan displaying system.

1. 1) Nabi, R. I., et al. J. Cell. Sci. 2015, 128, 2213-2219.

#13 - Structural changes in liver glycogen induced by diseases

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Glycogen functions as a short-term energy reserve and plays a vital role as a glucose buffer *in vivo* for maintaining blood-glucose homeostasis. Liver glycogen is a hyperbranched glucose polymer, comprising individual β particles (~20 nm in diameter, each a single glycogen molecule), which can bind together to form aggregated α particles (> 40 nm in diameter). Glycogen functionality depends on its molecular structure, but its molecular structure can be altered by various diseases. For example, glycogen alpha particles in diabetic animals are more chemically fragile in the existence of H-bond disruptors than those in non-diabetic animals, which is consistent with the breakdown of blood-glucose homeostasis in diabetes. In addition, liver glycogen in animal models of liver fibrosis contains a higher proportion of β particles, with the glycogen containing fewer long chains than those in healthy animals. Transcriptomics and proteomics revealed a functional deficiency of mitochondria in the liver of fibrosis animal models, which may lead to changes in glycogen structure. Several drugs can restore the molecular structure of liver glycogen in some diseases, such as diabetes, by regulating enzymes involving in glycogen metabolism. These findings have implications for finding a novel type of drug target for diseases that lead to liver dysfunction.

POSTERS

#14 - The Immune System is Far Sweeter Than You Think: Truncated N-glycans Dominate the Immunopeptidome

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Immunopeptidomics, the study of immunopeptides presented on the cell surface by major histocompatibility complex class I and II (MHC-I and -II) molecules, is a rapidly emerging field of research offering new insights into how our immune system recognises self/non-self in health and disease. While we recently discovered that *N*-glycosylation is a dominant feature of immunopeptides presented via MHC-II by infected dendritic cells (Parker et al., 2021), the extent of *N*-glycosylation of the human immunopeptidome remains unstudied. To this end, we have systematically re-interrogated a valuable collection of MHC-I and -II immunopeptidomics LC-MS/MS datasets with a focus on identifying previously overlooked immunoglycopeptides distinguishable by their characteristic oxonium ion profile and other glycopeptide fragment ions detectable using tailored Byonic-based search strategies and in-house software. Interestingly, we found strong evidence for the wide-spread presence of *N*-glycosylated immunopeptides within both the MHC-I and MHC-II systems from diverse cellular and tissue origins (e.g. human monocytic and B-lymphoblastoid cell lines and colorectal cancer tissues) and across different patho-physiologies (e.g. cancer and infection). The MHC-II immunopeptides showed a particularly high frequency of *N*-glycosylation in some datasets accounting for more than 20% of all detected immunopeptides, while MHC-I datasets generally displayed less frequency of immunoglycopeptides. Notably, and in line with our previous report, a significant proportion of the detected MHC-II immunoglycopeptides carried highly truncated chitobiose core ($GlcNAc_{1-2}Fuc_{0-1}$) and paucimannosidic ($Man_{1-3}GlcNAc_2Fuc_{0-1}$) *N*-glycans while fewer oligomannosidic ($Man_{5-8}GlcNAc_2$) structures were identified. Strikingly, no complex *N*-glycans were identified in any of the re-interrogated MHC-I and -II immunopeptidomics datasets suggesting that antigen processing is accompanied by extensive glycan trimming before or during MHC presentation. Collectively, these findings highlight that *N*-glycosylation is a dramatically overlooked yet prevalent and likely biologically important feature of the immunopeptidome that urgently demand further investigation to allow for a better understanding of our immensely complex immune system.

1. Parker, R., et al. (2021). Mapping the SARS-CoV-2 spike glycoprotein-derived peptidome presented by HLA class II on dendritic cells. Cell Rep 35(8): 109179.

#15 - Characterising the subcellular glycoproteome during a time-course infection of influenza A virus

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Influenza A virus is responsible for substantial morbidity and mortality, particularly amongst children, pregnant women, and the elderly. Developing our understanding of viral-host protein interactions is essential given that current therapeutic strategies remain insufficient in combatting this disease.

Perturbations of the host proteome during influenza A virus infection are well established at a global level, however little attention has been given towards the host glycoproteome. The sialidase activity of the influenza glycoprotein neuraminidase, and interactions between viral-host proteins are thought to perturb glycosylation of host glycoproteins during infection.

We developed a LC-MS/MS based protocol using tandem mass tag (TMT) labelling to identify and quantify the dynamic changes in protein abundance, and glycosylation throughout the course of infection with two influenza A virus strains representative of the two subtypes predominating infections in humans: the H1N1 virus A/PR8/8/34 and the H3N2 virus A/X31.

We applied this protocol to organelle, nuclear, cytosolic, and secreted protein fractions of infected human A549 cells to characterise these changes at the subcellular level, and to discern strain-specific host responses.

#16 - Multi-omic analysis of plasma-derived extracellular vesicles in breast cancer

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Breast cancer was the most diagnosed cancer worldwide in 2020. Current imaging-based methods for monitoring treatment response lack molecular sensitivity and accuracy. Liquid biopsy is an emerging technology used to detect disease markers in plasma, with greater sensitivity and potential to target protein glycosylation changes implicated in breast cancer [1]. A suitable molecule for plasma liquid biopsy are abundant, biological nanoparticles called plasma-derived small extracellular vesicles (sEVs), containing proteins and glycoconjugates (N-glycans, polysialic acid (polySia), glycosphingolipids (GSLs) and glycosaminoglycans (GAGs)).

This project aims to determine proteomic and glycomic sEV signatures specific to breast cancer patients as a proof-of-concept for plasma liquid biopsy. Three sEV isolation methods (ExoQuick, ultracentrifugation and size exclusion chromatography (SEC)) were compared to determine which method obtains an enriched sEV population with minimal plasma (non-sEV) protein contamination. Using these methods, sEVs were isolated from healthy individuals (n = 2) and breast cancer patient (n = 2) plasma, then characterised using nanoparticle tracking analysis and Qubit protein assay. To investigate the sEV proteome, de-N-glycosylated peptides were analysed by nanoLC-MS/MS. To investigate the sEV glycome, multi-glycomics was used to analyse N-glycans, polySia, GAGs and GSLs. Enzymatically released glycans were analysed using HPLC (polySia and GAGs) and PGC-LC-MS/MS (N-glycans and GSLs).

Results demonstrated different isolation methods produced different sEV proteomes and glycomes. Overall, each method produced sEV proteomes containing sEV and non-sEV proteins, where SEC yielded the purest sEVs. The N-glycome differed between each method. Overall, 64 N-glycans were identified across complex, hybrid and oligo mannose types. Complex N-glycans were most abundant across all samples. Surprisingly, polySia, considered a predominately CNS component, was present in healthy and cancer patient sEVs. Fewer changes were observed in GAG and GSL glycomes. These multi-omic findings illustrate different sEV isolation methods result in varying molecular profiles, highlighting the challenges associated with analysing plasma-derived sEVs.

1. [1] Christiansen, M. N., Chik, J., Lee, L., Anugraham, M., Abrahams, J. L. and Packer, N. H. (2014) 'Cell surface protein glycosylation in cancer', Proteomics (Weinheim), 14(4-5), pp. 525-546.

#17 - High enzyme activity of plasma N-acetyl-β-hexosaminidase is a novel marker of poor outcome of colorectal cancer patients

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Colorectal cancer (CRC) is a prevalent and deadly type of cancer. CRC prognostication is still based on invasive colonoscopy and subjective histopathological tests of tissue biopsies. New less invasive and objective tests are therefore required to determine CRC patient survival chances to allow for early intervention and better patient management. We have recently discovered that paucimannosidic glycans (PMGs, $Man_{1-3}GlcNAc_2Fuc_{0-1}$) are significant signatures in various human immune cell types and across human cancers including CRC. We have also shown that PMGs are synthesised via an alternative biosynthetic pathway by N-acetyl-β-hexosaminidase (Hex) isoenzymes secreted by cancer and immune cells into blood. While associations between PMG and CRC prognosis have been reported, the prognostic value of Hex remains untested. Using a simple fluorescent-based substrate assay on

plasma from 139 CRC patients spanning stage I (n = 34), stage II (n= 40), stage III (n = 38), and stage IV (n= 27) and aged/gender-matched 38 healthy donors, we here show that the Hex enzyme activity in plasma of CRC patients is significantly higher than in plasma from heathy donors (p = 0.002, t-test, unpaired, two-tailed). Notably, we show that patients with high plasma Hex activity have significantly lower five-year survival rate compared to patients displaying low plasma Hex activity (Log-rank test, p = 0.0023). Supporting the link between Hex and CRC, we demonstrate that cultured CRC cells (LIM2045) treated with a pharmacological Hex inhibitor display less metastatic behaviour compared to untreated cells across several functional assays, indicating that Hex and/or downstream PMG-carrying glycoproteins are involved in key tumour-associated processes. Conclusively, we propose that Hex holds a considerable translational potential as a novel easy-to-monitor prognostic marker and represents a drugable therapeutic target against CRC.

#18 - A comprehensive glycoproteomic approach to identify melanoma-specific neoantigens

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Melanoma is the rarest and deadliest of all skin cancers combined. Although relatively rare, representing only 11% of all cancer diagnoses in 2022, melanoma is the third most diagnosed cancer in Australia. However, systematic melanoma therapy is still very limited. A promising alternative to cancer immunotherapy involves a focus on tumor neoantigens. Neoantigens are considered important targets for cancer immunotherapy because they are not expressed in normal cells and they are highly immunogenic, making melanoma an ideal model due to its high mutational burden. Furthermore, because these mutated, immunogenic peptides are not expressed in normal cells, one could expect a neoantigen-based immunotherapeutic approach to be well tolerated and have negligible side effects. Although the characterization of melanoma-specific immunopeptides has been attempted for more than 30 years, the identification of cancer-specific neoantigens remains a challenge. The objective of this study is to identify novel neoantigens specific to melanoma using a mass spectrometry-based glycoproteomic approach. In this study, MHC-peptides are obtained from melanoma cell lines by generating cell lysates and capturing MHC-peptide complexes from the cell lysates using immune-affinity purification and a mild acid elution protocol. Subsequently, the peptides are separated by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) prior to analysis by mass spectrometry. Identification of neoantigens will accelerate the development of precision cancer immunotherapy. This research project will serve as a platform to identify target neoantigens that can be used to develop new vaccines for the treatment of melanoma.

#19 - Effects of N-Acetylglucosaminyltransferase GCNT3 down-regulation on the pro-metastatic, adhesive properties of human adenocarcinoma cells

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An important step in the process of hematogenous metastasis is the adhesion of circulating tumor cells (CTCs) to the vascular endothelium. The carbohydrate antigens sialyl-Lewis A (sLeA) and sialyl-Lewis X (sLeX) are significantly involved in this process. sLeA and sLeX are bound to glycoproteins, proteoglycans or glycolipids on the surface of tumor cells and are canonical ligands for E-selectin expressed by vascular endothelial cells. Based on preliminary data, we hypothesized that the glycosyltransferase GCNT3 is critically involved in the synthesis of glycan scaffolds for sLeA/X by synthesizing the core 2 structure of *O*-GalNAc glycans in human gastrointestinal cancer. To test this hypothesis, GCNT3 was knocked down in three sLeA/X-positive human gastrointestinal adenocarcinoma cell lines (HT29, GC5023 and Paca5061) using shRNA. All tested GCNT3 knockdown

(KD) derivatives indeed showed a moderately decreased static E-selectin binding capacity, whereas the concurrent effects on the total sLeA/X levels at the cell surface were variable. Of note, dynamic tumor cell adhesion on E-selectin was reduced only in case of HT29 cells while adhesion on endothelial cells was not altered in either cell line. Effects on the proliferative behavior in conventional 2D as well as 3D conditions were also variable but tumor cell migration was moderately impaired in all tested cell lines. Analyses of GCNT3 KD-induced changes in the glycosphingolipid composition revealed cell line-specific changes in single structures but, when altered, they always increased in the GCNT3 KD. Analyses of the *N*- and *O*-glycan composition are still ongoing. Together, the results suggest that further E-selectin ligands beyond sLeA/X exist and that different ligands might function for static vs. dynamic E-selectin binding. GCNT3 is involved in the synthesis of glycans that partially determine static E-selectin binding and migratory capacity of human gastrointestinal adenocarcinoma cells.

#20 - The serum *N*-glycome of septic shock patients

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Sepsis, the excessive host response to bloodstream infections, remains a prevalent condition with an alarming mortality, in part, due to an incomplete understanding of the molecular mechanisms underpinning the disease onset and progression. Building on our recent finding documenting that aberrant serum *N*-glycosylation is a feature bacteraemia, the presence of pathogenic bacteria in blood, relative to non-infected blood [1], we here set out to unravel the key molecular perturbations associated with sepsis by exploring the serum *N*-glycome of critically ill individuals experiencing septic shock conditions. To this end, the serum *N*-glycome of 10 septic shock patients infected with different pathogens (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*) was profiled using a robust PGC-LC-MS/MS approach to quantitatively map the *N*-glycan fine structures with isomer resolution. In total, 47 *N*-glycan isomers comprising primarily complex-type core fucosylated, bisecting *N*-acetylglucosamine (GlcNAc), and α2,3-/α2,6 sialylated *N*-glycan structures were identified in the septic shock sera, which were then compared to serum *N*-glycome data of 10 healthy donors and 27 bacteremic patients acquired using identical conditions. Interestingly, the serum *N*-glycome of septic shock patients exhibited discriminatory traits allowing accurate stratification from healthy donors and bacteraemic patient group as assessed using hierarchical clustering and principal component analysis. Upon closer interrogation, the septic shock serum *N*-glycome was found to display unique molecular signatures including, most prominently, a consistent depletion of oligomannosidic-type glycans across all septic shock patients when compared to healthy donor and bacteremic sera. In summary, this study documents that unique serum glycophenotypes associated with sepsis implicate serum glycans as new potential players in a yet-to-be fully understood disease process causing or resulting from sepsis.

1. [1] Chatterjee, S, Kawahara, R, Tjondro, HC, Shaw, DR, Nenke, MA, Torpy, DJ & Thaysen-Andersen, M 2021, 'Serum N-Glycomics Stratifies Bacteremic Patients Infected with Different Pathogens', Journal of Clinical Medicine, vol. 10, no. 3, p. 516.

#21 - Unravelling the impact of respiratory viruses on the glycome of mammalian cells

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Every cell in our body is decorated with complex carbohydrates known as glycans which are N-linked or O-linked to proteins as a part of post-translational modifications, or linked to lipids. Glycans play multiple biological roles important for protein folding and stability, cell-cell and cell-matrix interactions, and for the physical and structural integrity of cells. It is also well established that viruses (e.g. influenza) utilise host cell glycans for the attachment and entry into cells. However, little is known about possible changes in host cell glycome upon viral infection and their consequences on antiviral immune response. Glycome modifications have been well investigated for non-infectious diseases (e.g. cancer, diabetes), and altered glycosylation has been found to be associated with disease pathogenesis. Our study is designed to investigate the possible changes in host glycome triggered by infection with two pathologically important respiratory viruses: human parainfluenza (HPIV) and human respiratory syncytial virus (RSV). Children under the age of five and immunocompromised individuals are two populations severely affected by HPIV and RSV infection globally. We hypothesize that glycome changes occur in host cells upon viral infection, to prime the infected cell microenvironment to be more susceptible to antiviral innate immune response. We are using differentiated primary human airway epithelial cells (HAE) to truly recapitulate the consequences of viral infection on the host cell glycome. Mass spectrometry (MS) coupled with liquid chromatography has been the method of choice in the last few decades for glycomics, glycoproteomics and glycolipidomics. Moreover, we are currently working on to establish a methodology to compare and validate our results of underivatized glycans with derivatized (permethylated) glycans in a setting of PGC-ESI-MS/MS, MALDI-TOF MS and C18-ESI-MS/MS. Our research will act as a platform to identify glyco-based markers of viral infection that could possibly improve the innate immune response against respiratory viruses.

#22 - Identification of a new class of free oligosaccharides in Salmonella typhimurium hot phenol extracts

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Glycans and glycoconjugates are key structural molecules in bacteria. They are central to microbial interaction and adaptation to their environment, governors of physiological traits such as size and shape and are a common component of biofilms. While the functional contribution of bacterial glycoconjugates is firmly established in the literature, the scope of free oligosaccharides (fOS) is comparatively underexplored. fOS is known to play an important role in maintaining intracellular homeostasis in the form of periplasmic glucans. These fOS, consisting of as many as 40 glucose units linked in large branched linear or cyclic structures, are regulators of the osmotic balance in the periplasmic space[1]. A similar function has also been attributed to fOS released from lipid linked oligosaccharides derived from bacterial protein glycosylation systems[2].

Here we present preliminary mass spectrometry (MS)-based characterisation of a class of free oligosaccharides distinct from periplasmic glucans, detected in hot phenol extracts of Salmonella enterica serotype typhimurium. MS-fragmentation of these fOS revealed they are composed of HexNAc-HexNAcA-dHexNAc repeats with the major species constituting a dodecasaccharide. By altering our enrichment strategies we were able to gain insight into the macromolecular arrangement of these glycans as well as showing they are the target of a range of chemical modifications including methylation and O-acetylation. To the best of our knowledge this is the first study to characterise these glycan species in S. typhimurium, paving the way for subsequent studies aimed at identifying the intracellular functionality of these glycans in addition to the biosynthetic pathways responsible for their synthesis.

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2. 2. Nothaft, H., Liu, X., McNally, D. J., Li, J., Szymanski, C. M. 2009. Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. Proc Natl Acad Sci USA 35:15019-24

#23 - Development of biomimetic growth factor delivery systems

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Tissue engineering and regeneration is an inter-disciplinary field of research that combines principles from both biology and engineering. While the use of biomaterials has long been associated with this field of research, more recently there has been a paradigm shift for the modern biomaterial to be biomimetic, through replication of the *in vivo* situations they are trying to substitute. Growth factors and their use as a therapeutic is of great interest in tissue regeneration applications however, to achieve a beneficial response, appropriate administration is required. Furthermore, due to biological heterogeneity of these structures, their low abundance, and difficulty in isolation from mammalian tissues there is a need to develop an alternative source of these biomimetic materials.

This study aims to develop materials that mimic biological sulphated sugar structures, known as glycosaminoglycans, that protect and deliver growth factors *in vivo*. It was hypothesised that by adjusting structural variables, the specificity and affinity of these biomimetic materials towards different growth factors could be modulated.

Results demonstrated changes in material structure modulated the specificity and affinity of the biomimetic materials towards fibroblast growth factor(FGF)2 and FGF7, along with the ability to signal FGF2. Additionally, the biomimetic materials were demonstrated to enhance epithelial cell migration and enhance formation of an epidermis in an organotypic skin model.

This study demonstrated the ability to develop materials that mimic the biological function of growth factor binding molecules glycosaminoglycans.

#24 - Protein Glycosylation in Established and Emerging Human Viral Respiratory Diseases

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The Covid-19 pandemic has led to many studies looking into the glycobiology of the SARS-CoV-2 virus, in particular its trimeric spike protein. In the meta-analysis of SARS-CoV-2 research over the course of the pandemic, many developments, both in our understanding of the SARS-CoV-2 trimeric spike protein's glycobiology, as well as the techniques and methods in analysing the glycoproteome of the spike protein, allow us to develop a better comprehension in developing novel methods in protecting us from the pandemic causing respiratory virus.

#25 - Fucose signatures in peripheral blood glycoproteins are associated with reduced clinical benefit of immune-checkpoint inhibitors in metastatic melanoma

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The clinical success of immune-checkpoint inhibition (ICI) in melanoma has confirmed the merit of therapeutic strategies that boost the immune system to counteract cancer. However, only about half of patients derive a long-lasting benefit. While elevated PD-L1 expression and tumor mutational burden correlate with the likelihood to benefit from ICI therapy in some indications, these biomarkers have

shown poor predictive performance in metastatic melanoma. By applying InterVenn's glycoproteomics platform to pretreatment plasma samples from metastatic melanoma patients receiving anti-PD-1/anti-CTLA-4 therapy, we identified a panel of biomarkers that differentiate patients likely to benefit from those unlikely to benefit from ICI. A laboratory-developed test based on these findings, DAWN™ IO Melanoma, was developed and has been introduced into the market.

Here, we report our analysis of the glycosylation patterns from 205 patients with metastatic melanoma who received ICI therapy. We identified a fucosylation signature in *N*-linked glycoproteins that identified individuals unlikely to benefit from ICI therapy. To test this observation, we engineered site-specific glycosylation features that represent the average number of specific monosaccharides at a given site, weighted by glycopeptide occupancy. Of 52 fucose-dependent features, 12 were associated with benefit from ICI therapy based on univariate Cox regression analysis (FDR <0.05). Two features were retained in a repeated cross-validated LASSO-regularized Cox regression model on a training set consisting of 40% of the cohort, yielding a hazard ratio (HR) of 5.1 (p=3e-05). A validation set consisting of 30% of the cohort was used to tune model hyperparameters. When applied to the remaining 30% of the cohort, this tuned model resulted in a HR of 2.6 (p=3e-02), indicating that fucose-dependent features stratified patients in groups differing in the likelihood of deriving benefit from ICI therapy, such that patients with a risk score exceeding the selected threshold were nearly three times less likely to respond.

#26 - PeptideRT - A Deep Learning Method for Peptide Retention Time Prediction

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Development of machine learning methods to identify peptides in mass spectrometry data constitutes a breakthrough in computational proteomics. Deep-learning technologies have provided feasible solutions to estimate both peptide physical and separation properties with better performance while requiring less computational resources.

Here, we present a deep-learning framework for peptide retention time prediction, called PeptideRT. We compared multiple architectures including convolutional neural networks, recurrent neural networks and attention mechanisms with an additive-layer inclusion approach to observe performance. PeptideRT uses human serum binary peptide sequence information and physicochemical descriptors as input for these neural network architectures to demonstrate its capability to predict retention times. By training on a sample set of 8,678 unique peptide sequences, we were able to develop a novel architecture with a Multi-Head Attention Mechanism which gives best results at ~97% (R2 and R2-Adjusted), showing that data fits the model well without overfitting. Predicted and actual retention time values have a strong Pearson's correlation at ~0.987. Furthermore, our model increases both the sensitivity and reliability of peptide identifications compared to existing methods. Currently, we are developing a Python library for users to process peptide sequence data and predict their retention properties.

Continued advancements in statistical and computational methods are enabling more sophisticated data analysis processes. It is expected that deep-learning will have a profound impact and tools such as PeptideRT can serve to be useful in peptide retention time prediction and the identification of important descriptors. Ultimately, access to such tools and high-quality annotated data collections will facilitate deep-learning applications, which can reduce false peptide identifications whilst improving quality control for spectra matching algorithms.

#27 - The non-conventional Hex-mediated *N*-glycan truncation pathway is highly active in GM-CSF-differentiated macrophages and diminished upon mTOR inhibition

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Macrophages are heterogeneous, dynamic and plastic innate immune cells critical in our first lines of response to pathogens. We recently reported that human macrophages and their monocytic precursors display an unusual *N*-glycophenotype comprising rich protein paucimannosylation ($Man_{1-3}GlcNAc_2Fuc_{0-1}$) (Hinneburg *et al.,* 2020). However, the dynamic utilisation and potential regulation of the non-conventional *N*-acetyl-β-hexosaminidase (Hex)-mediated truncation pathway responsible for paucimannosidic protein formation in neutrophils (Ugonotti *et al.*, 2022) remain unstudied in the context of macrophage differentiation and polarisation. Herein, we explore using label-free *N*-glycomics and sensitive proteomics the *N*-glycosylation landscape and glyco-enzyme expression of various sub-populations of macrophages (M0, M1, M2) upon GM-CSF-driven differentiation and cytokine-mediated polarisation from primary monocytes derived from healthy donors. Since GM-CSF reportedly activates mTOR signalling, we also investigated if Torin-1, a known mTOR inhibitor, can reverse the glyco-phenotypic changes observed upon GM-CSF differentiation. Firstly, *N*-glycomics showed a considerable elevation of paucimannose in the GM-CSF-differentiated macrophages (relative to their monocytic precursors) and a surprisingly similar *N*-glycome signature of the M0-M2 sub-populations indicating a consistently high utilisation of the Hex-mediated truncation pathway following GM-CSF-differentiation. Interestingly, upon Torin-1 treatment, all macrophage sub-types displayed a prominent reduction in paucimannosylation while maintaining viability and their characteristic morphology suggesting an association between the mTOR pathway and the Hex-mediated truncation pathway. Importantly, proteomics demonstrated not only raised Hex levels in all macrophage sub-populations upon GM-CSF differentiation, but also showed a corresponding reduction in the Hex expression upon Torin-1 treatment adding support for the suggested relationship between mTOR activation/inhibition and modulation of the Hex-mediated truncation pathway. Collectively, data from this system-wide -omics study point to an interesting and hitherto undescribed link between the mTOR pathway and protein paucimannosylation formation and regulation in differentiating macrophages that are critical to maintain health and fight disease.

1. Hinneburg H, et al., 2020 High-resolution longitudinal N- and O-glycoprofiling of human monocyte-to-macrophage transition. Glycobiology 2022 Mar 30;32(3):218-229. doi: 10.1093/glycob/cwab108.
2. Ugonotti J, et al., 2022 N-acetyl-β-D-hexosaminidases mediate the generation of paucimannosidic proteins via a putative noncanonical truncation pathway in human neutrophils. Glycobiology 2020 Aug 20;30(9):679-694. doi: 10.1093/glycob/cwaa020

#28 - Towards the Degradation of Galectin-3 using Proteolysis Targeted Chimeras (PROTACs)

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From previous studies[1], it is clear that the use of Proteolysis-targeting chimera (PROTAC) molecules can result in the effective degradation of proteins of interest (POI). PROTAC techniques involve the exploitation of normal protein degradation essential for cellular maintenance, whereby the system is hijacked to specifically degrade target proteins.

Work is underway within the Institute for Glycomics to synthesise novel PROTAC molecules to achieve successful proteolysis of the Galectin-3 (Gal-3) protein. Recent investigations[2] have revealed that not only is overexpression of Galectin-3 within cancer-cell lines commonplace, but is also intimately associated with increased tumour aggressiveness, metastatic potential, induced immunological tolerance for malignancies, and ultimately can cause multi-drug resistance to current anti-cancer therapeutics.

To achieve an effective PROTAC design the molecule must provide high affinity binding to both Gal-3 and a suitable ubiquitin ligase (Cereblon & von Hippel-Lindau – targeted in this project), and maintain these interactions whilst not inhibiting the overall ubiquitination (or tagging for destruction) process.

Results from the following aspects of the project will be presented: (i) synthetic design of a potent Gal-3 inhibitor/ligand modified to enable attachment of a ubiquitin ligase ligand; (ii) Molecular Dynamics simulations on the binding of the modified Gal-3 inhibitor/ligand to the Gal-3 CRD; (iii) synthesis of the modified Gal-3 inhibitor/ligand; (vi) competition STD NMR experiments to assess the impact of the linker arm on the binding of the Gal-3 inhibitor/ligand to Galectin-3 (POI).

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[2]. Bum-Erdene, Collins, Hugo, Tarighat, Fei, Kishor, Leffler, Nilsson, Groffen, Grice, Heisterkamp, Blanchard. 'Novel Selective Galectin-3 Antagonists Are cytotoxic to Acute Lymphoblastic Leukemia'. *Journal of Medicinal Chem*istry, 65 (2022) 5975-5989.

SPEED TALKS 2

29 - A comprehensive glycomics data repository workflow by strengthening collaboration between UniCarb-DR and GlycoPOST

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In the life sciences, recently many public data repositories have been rapidly developed to store datasets submitted by researchers and to promote sharing of such data under the FAIR (Findable, Accessible, Interoperability, and Re-usable) data principles. In the glycosciences, the Minimum Information Required for A Glycomics Experiment (MIRAGE) proposes a set of standard guidelines when reporting qualitative and quantitative results of glycomics experiments including mass spectrometry, glycan arrays, liquid chromatography, etc. Our lab has been developing the GlyCosmos project, which offers two major public data repositories, UniCarb-DR and GlycoPOST, to allow researchers to submit their mass spectrometry-based experimental results following the MIRAGE guidelines. UniCarb-DR can visualize the MS/MS spectra data submitted by researchers in GlycoWorkbench format (.gwp), and users can browse each of the spectra and glycan structures in their web browsers. In addition, by providing versatile data search functionalities, it enables users to search specific spectra data which fulfills various conditions, e.g. it is reported in a specific publication or contains the monosaccharides having specific compositions. GlycoPOST, on the other hand, can accept various MS experiment-related resources from researchers including the raw data from mass spectrometers. The researchers can make their data public after an embargo period; it thus allows them to configure their data publication date freely. However, this functionality is not available in UniCarb-DR. In this study, we tried to enhance the integration of these complementary data repositories by unifying the data submission flow and enabling cross-referencing. As a result, we propose a new comprehensive glycomics data repository workflow combining the advantages of these two repositories, making it easier for users to submit mass spectrometry data. These repositories are freely available on the GlyCosmos Portal website.

#30 - Extraction conditions significantly impact the resulting proteome, glycome and glycoproteome

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The extraction of glycoproteins from complex biological mixtures is a crucial first step in any (glyco)proteomic workflow. The efficacy of the extraction method is known to directly impact the subsequent depth of proteome analyses, and extraction conditions must be optimised for specific biological systems. Numerous extraction protocols have been developed for proteomics studies, however, the impact that these extraction conditions have on the reproducibility and comparability of glycomics and glycoproteomics studies remain unclear. This is especially relevant for studies using patient specimens (e.g., tissue biopsies). This work aims to identify, validate, and quantitate the impact that different extraction conditions have on the glycome and proteome results, using a human melanoma cell line model. We also aim to assess how these extraction conditions, or combinations thereof, can be utilised to increase the depth of coverage in glycoproteomics studies.

Five widely used extraction conditions (RIPA, Triton X-100, TRIS buffered saline, urea, and a commercial “TriPrep” kit) were used to extract (glyco)proteins from a human melanoma cell line. The

proteome and glycome were analysed using Reversed Phase C18- and porous graphitised carbon (PGC)-nanoLC-ESI-MS/MS technologies, respectively. The different extraction conditions resulted in significantly different proteome and glycome profiles. Of the 4785 protein groups identified using label-free quantitation, we determined that 65% were differentially represented across all extraction conditions. We identified protein subgroups that were preferentially recovered in certain conditions and correlated these to specific subcellular locations. Our glycomics analyses revealed that the relative quantitative glycome profile was extraction condition dependent. These variabilities were evident in most glycan subtypes but were especially significant for sialylated N-glycans. Future work will ascertain whether protein biophysical properties can be used to guide preferential extraction condition(s) to improve the efficiency of (glyco)proteomic investigations.

#31 - Dual search strategy for identification of glycopeptide with multiple N- and O-glycans

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Mass spectrometry (MS)-based glycopeptide identification usually starts from peptide sequences or glycan residues. For peptide-first strategy, the most commonly used Byonic provides a confident peptide sequence and deduces a total composition with an uncertain glycan structure and localization. In contrast, the glycan-first strategy, such as pGlyco3, traces first the Y ions and/or glycan residues to deduce the probable composition and then the peptide sequence. While both search strategies may lead to identifying the same glycopeptides, especially those affording sufficient MS2 ions, the eventual assignments provided may be different. Often, part of the identified glycopeptide is unreliable when the necessary Y ions or peptide b/y/c/z ions are missing. Moreover, site-specific O-glycosylation assignment relies on the glycan-retaining c/z ions of EThcD-MS2. O-pair, another peptide-first software, adopts an open search strategy to first identify the peptide based on HCD-MS2 and then pair the positive spectrum match to its corresponding EThcD-MS2. pGlyco3, on the other hand, merges the HCD and EThcD-MS2 pairs first before searching. Both O-pair and pGlyco3 allow and score the reliability of localizing the O-glycan but can only handle search for N- and O-glycopeptides separately, not simultaneously. Only Byonic, searching the HCD and EThcD-MS2 data independently, allows both N- and O-glycosylation modification search either separately or simultaneously but typically consumes a long searching time with much uncertainty of the deduced glycan compositions. How O-pair and pGlyco3 handle the glycopeptides with multiple N- and O-glycans and whether paired or merged MS2 is a better strategy for confident identification and localization of multi-glycosylated peptides are unclear. We present here our systematic appraisal of this dual search strategy for N-, O-, and multiply glycosylated peptides from a variety of glycoprotein sources and demonstrate how a dual search strategy helps reduce false positives and negatives compared to using only one but not the other software.

#32 - GSS: a web tool to simulate signaling pathways involving glycosylation

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To date, variouos mathematical models have been developed to elucidate the mechanisms of signal transduction. However, existing models of signal transduction rarely contain glycosylation information in spite of the fact that many proteins are modified by glycans, and it is known that deficiencies and mutations in glycosylation can affect signal activation.

Therefore, we have developed an online tool called GSS (Glycan Signaling Simulation) to simulate the effects of glycosylation in signaling pathway.

Moreover we attempted to validate our tool by building a model of Notch glycosylation and analyzing it.

GSS has a user-friendly graphical interface, mainly providing functionalities to integrate glycosylation using GlycoSim (https://glycosim.rings.glycoinfo.org) into existing signal transduction models, simulating the built model and performing parameter estimation to fit the parameters of the model with experimental data.

We imported a basic Notch signaling model by Sivakumar2011 from the BioModels database (http://www.ebi.ac.uk/biomodels/) into GSS and further added the glycan biosynthesis of two monosaccharides by OFut1 and Fringe. The model was constructed to only include reactions involving the Notch protein and relevant glycotransferases by removing irrelevant reaction elements to reduce the number of model parameters.

Parameter estimation methods were executed based on experimental data by Taylor2014 [1] using the concentration of glycosylated Notch modified with an O-Fucose by OFut1 and O-GlcNAc by Fringe at EGF-12.

As a result, we could obtain parameters that resulted in simulations of this Notch glycosylation model that almost perfectly matched the experimental data (Fig. 1).

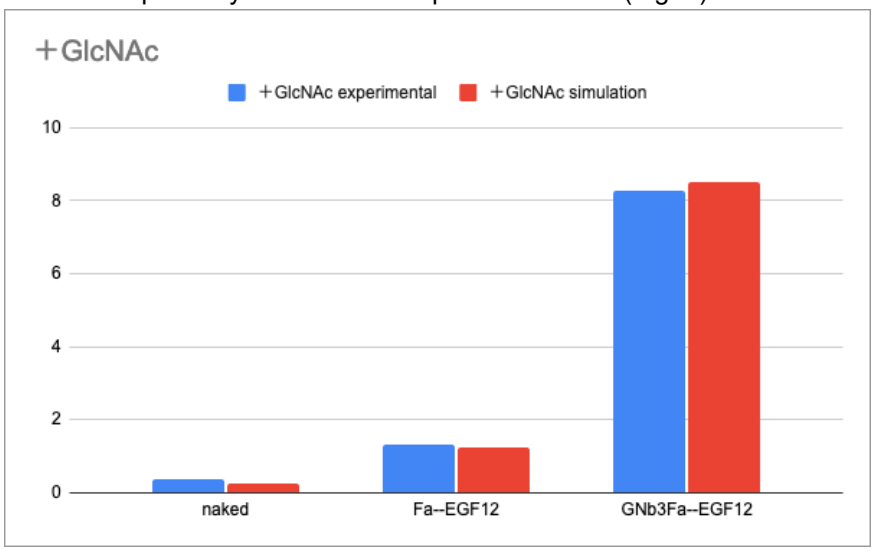

FIg1. Comparison of experimental and simulated data.

This was the first time we were able to show that GSS is able to build models and simulate signal transduction that includes glycosylation. We will continue to develop more complex glycan biosynthesis models and improve the functionality of GSS by incorporating high-performance parameter estimation algorithms.

GSS is currently available at https://glycosim.rings.glycoinfo.org/signaling

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#33 - Development of a database of glycosylation reaction parameters, GlycoParaDB

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Our lab has been developing an online glycosylation simulation tool called GlycoSim, which can predict glycosylation pathways, construct mathematical models, and perform simulations. We have been using GlycoSim to estimate O-glycan profiles of mouse embryonic stem (ES) cells, embryoid bodies (EB) and extra-embryonic ectoderm (ExE) cells, based on data from the experiments of Nairn et al[1]. However, when performing glycan synthesis simulation, the parameter values of the rate coefficient of enzyme reactions (kf), the dissociation constant between glycan substrate and enzyme (Km), and the dissociation constant between sugar nucleotide and enzyme (Kmd) are required, but these parameter values are unknown. Therefore, we decided to estimate these unknown parameter values and determine the range of each reaction parameter value based on the experimental data and parameter estimation methods. The objective is also to create a database of these determined parameter values

and to make it publicly available. The mathematical model of O-glycosylation was constructed using GlycoSim, and parameters were estimated using COPASI, an application software for simulation and analysis of biochemical networks and their dynamics. This was repeated numerous times to obtain a distribution of parameters, and based on the best estimated parameter values, simulation of O-linked glycan biosynthesis was performed. In our simulation results, we could reproduce the same glycan profiles as the experimental results in all the ES, EB, and ExE cells. The determined parameter values were converted to Resource Description Framework (RDF) format, and a database, GlycoParaDB, was created to make the parameter values available. In the future, we will conduct simulation analysis of the biosynthesis of N-linked glycans in mouse ES cells, determine the reaction parameter values, and supplement our newly created database. GlycoParaDB is being considered for inclusion in the glycoscience portal GlyCosmos[2].

1. [1] Nairn,A et al.Regulation of glycan structure in murine embryonic stem cells.Journal of Biological Chemistry.287(45):337835-37856,2012
2. [2]GlyCosmos. https://glycosmos.org

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

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

#35 - Understanding storage associated changes in platelet components using glycomics

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Platelet transfusions are lifesaving treatments for various bleeding patients ranging from cancer post-chemotherapy and cardiac patients. However, they are in high demand. Currently, platelets are stored at 22°C for a maximum of 7 days, but they deteriorate gradually developing what is known as platelet storage lesions. While there have been many metabolomic, proteomic and lipidomic studies conducted analysing stored platelets there is still much left to learn to understand how platelet quality is effect during storage. Currently, no laboratory marker has been identified to assess stored platelet quality, hence their performance and survival *in vivo* cannot be assessed before transfusion. As the platelet's ability to perform many of its adhesive and immune functions comes from its many glycoproteins it is possible that they might be a suitable quality marker.

However, the glycome and glycoproteome changes that occur during storage and how this impacts their functionality remain unknown. To answer these questions, we have been investigating the platelet glycome and (glyco)proteome of 6 donors across three time points throughout the storage cycle (days 1, 5 and 7). The storage-dependent platelet N- and O-glycome have been investigated using well-established porous graphitized carbon (PGC)-nanoscale liquid chromatography (nanoLC)-Electrospray (ESI) tandem Mass Spectrometry (MS/MS) workflows while the platelet (glyco)proteome has been captured using reversed-phase (RP) nanoLC-Orbitrap MS/MS glycoproteomics workflows that included HILIC enrichment of glycopeptides. Sialic acid quantity and O-acetylation levels were determined using HPLC analysis after DMB-labelling.

The platelet glycome was successfully characterised to contain more than 120 different N-glycan structures with diantennary, sialylated N-glycans being the most abundant. Donor and time-dependent variations in the platelet glycome were being observed. The glycome information is currently used for ongoing glycoproteomics experiments to understand the protein-specific changes that can be derived from donor variability, processing, platelet activation and/or storage time.

#36 - Human cancer cells utilize various pro-adhesive ligands for endothelial adhesion *in vitro*

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One critical step of metastasis formation is the extravasation of circulating tumor cells into the metastatic organ's stroma to escape the adverse conditions existing within the bloodstream. This process requires the adhesion of tumor cells (TCs) to endothelial cells (ECs) via carbohydrate ligands binding to E-selectin and further endothelial cell adhesion molecules (CAMs). Our study aimed to characterize these glycan ligands more precisely.

We analyzed the endothelial adhesion of nine human TC lines using a laminar flow adhesion assay on human umbilical vein ECs (HUVECs). The TC lines were grouped into three subsets according to their canonical E-selectin ligands status expressing either sialyl-Lewis A and X (sLeA/X), sLeX only, or none of them. Adhesiveness of the TCs was compared after enzymatic, pharmacologic, chemical (including cleavage of sialic acid residues or glycoproteins or inhibition of *O*- or *N*-glycosylation) or antibody blockade treatment of the TCs or ECs, respectively. The TCs were also screened regarding their glycosyltransferase expression profile.

Endothelial adhesion did not exclusively require the presence of sLeA and/or sLeX. Other (non-canonical) ligands must exist since all TCs were able to adhere on human ECs, including sLeA/X-negative TCs. However, two of the three sLeA/X-negative TCs additionally or fully depended on VCAM-1 for endothelial adhesion. Nearly all tested cell lines adhered via terminal α-2,3-sialic acid. The significance of *O*-GalNAc- and *N*-glycans varied among the cell lines tested. The sLeA/X double-positive subset showed glycoprotein-independent adhesion, so carbohydrate ligands on glycolipids should be considered as well in future studies. All cell lines of the sLeA/X-negative subset consistently lacked FUT3 and FUT7 expression as opposed to sLeA- and/or X-positive cell lines.

E-selectin and α-2,3-sialic acid largely determine endothelial adhesion of human TCs. Nevertheless, the interaction between endothelium and TCs remains a complex process that requires future studies for a better understanding.

#37 - Synthesis of rare L-hexoses

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L-Hexoses are a family of exceptionally rare, prohibitively expensive, and synthetically challenging sugars with great biological significance. Requiring access to L-iduronic acid building blocks for the construction of glycosaminoglycan mimetics, we have recently developed the "fluorine-directing effect" to rapidly access these compounds with high efficiency. Our strategy, which entails the stereoselective C-5 epimerisation of D-hexose derivatives, has been optimised for the construction of L-IdoA[1] and L-idose[2] glycosyl donors. Its success has also been understood with the aid of density functional theory (DFT) calculations. Herein we describe the extension of this chemistry to carve out new synthetic routes to other rare L-hexoses. These include the commercially unavailable L-altruronic acid, a component of the capsular polysaccharides of the pathogens *A. viridans* var. homi and *P. mirabilis* O10, and L-guluronic acid, a key component of alginates which are polysaccharides of considerable industrial importance.

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2. See, N.W.; Wimmer, N.; Krenske, E. H.; Ferro, V., A Substituent-Directed Strategy for the Selective Synthesis of L-Hexoses: An Expeditious Route to L-Idose. *Eur. J. Org. Chem.,* **2021***, 2021* (10)*,* 1575 – 1584.

POSTERS

#38 - The application of identification software tools towards precise and comprehensive glycoproteomics

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Identification of site-specific glycosylation by direct LC-MS/MS sequencing of intact glycopeptides in combination with identification software is nowadays the mainstream workflow in glycoproteomics. More and more software tools are continuously developed based on different strategies and algorithms that are suitable for identification of different modifications by different analytical workflows, eg. *N*- or *O*-glycosylation; step HCD or EThcD MS/MS approach. We have been using Byonic software for productive LC-MS/MS-based glycopeptide analysis for a long time. More recently, we have additionally evaluated the newly introduced pGlyco 3 as an alternative and probably better or complementary software for glycopeptide identifications. As presented here in this work, we set out to compare in details their *N*-glycopeptide identification results using single purified glycoproteins digested with both trypsin and chymotrypsin. Digestion by two proteases at the same time effectively introduces more semi-specific, non-specific and mis-cleavage possibilities, thereby increasing the computational challenges to precisely identify the correct glycopeptides. We applied the widely accepted or recommended cut-offs to filter the search results for manual verifications. By using a combination of approaches, we demonstrated that the overlapping subset of identification results by the two software tools would carry the lowest proportion of mis-assignments and hence highest level of accuracy. However, as would be expected, including results that were only identified by one of the software would provide a more comprehensive picture of glycoproteomics. More importantly, we have identified many pitfalls and common sources of misassignment or non-identification errors associated with protein samples digested by more than one proteases. A selection of how these would dramatically impact the identification results is presented here.

#39 - A strategy to discover functional *N*-glycosylation sites for protein stability

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N-glycosylation is particularly important for glycoprotein stability on account of its role in protein folding in the Endoplasmic Reticulum (ER) and stabilization of mature glycoproteins. However, the impact of site-specific N-glycosylation on protein stability has not been well explored, although it is important for fundamental glycoprotein biology as well as in biopharmaceutical production and quality control. Our study pursued a novel strategy using genetic perturbation of glycosylation together with thermal proteome profiling (TPP) and quantitative multiple reaction monitoring mass spectrometry (MRM-MS) proteomics, to discover N-glycosylation sites with functional roles on protein stability. We then used intrinsic tryptophan fluorescence and protein unfolding/folding with guanidinium titration to validate the impact of select N-glycosylation sites on protein stability.

#40 - Validation of purification protocol and high-performance liquid chromatography (HPLC) analysis for the determination of oligosaccharide composition in milk samples

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Milk is a complex mixture of lipids, proteins, carbohydrates and minerals not only providing optimal nutrition for the young, but also supporting the health and development of young mammals during lactation. The focus of this research is on free oligosaccharides. These compounds have been shown to play an important role in the development of the brain, maintaining a healthy gut and modulating the immune system. These components are highly species-specific and also change during lactation in order to accommodate the different needs of each mammalian species. Understanding the differences between the milk of different mammals supports our understanding of the role of these compounds during development.

In order to determine the oligosaccharide composition in milk, samples have to be partially purified to remove milk components that might interfere with the analysis. Seven different methods were tested utilising an internal standard to determine the recovery rate. The most efficient method was identified, further optimised and validated to achieve quantitative recovery of the oligosaccharides.

For the analysis, the partially purified oligosaccharides were labelled with 2-amino benzamide (2-AB) and separated on a HILIC HPLC column according to Albrecht et al.1 Herein we describe the validation of both the labelling reaction and subsequent HPLC analysis including the determination of the limit of quantification (LOQ), limit of detection (LOD), linearity and range as well as the precision and accuracy.

These methods were applied to investigate the milk of different pastoral animals such as sheep, cows, goats and deer.

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#41 - Regulation of Protein N-linked Glycosylation Site Occupancy.

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N-glycosylation is an essential co/post-translational modification of proteins in eukaryotes, which involves the transfer of glycan from lipid-linked oligosaccharide (LLO) to select asparagine side chains

in Asn-Xaa-Thr/Ser (Xaa≠Pro) sequons of newly synthesized polypeptides in the lumen of the endoplasmic reticulum. N- glycosylation directly affects protein folding and plays important roles in protein function, stability, solubility, secretion, resistance to proteases and temperature, and enhancing half-life. Interestingly, the importance of N-glycosylation is highly variable between different glycosylation sites. However, the factors that determine the efficiency of site-specific N-glycosylation are not well understood due to the lack of tools to quantify site-specific glycosylation occupancy. Here, we aimed to understand how control of LLO biosynthesis affected site-specific N-glycosylation occupancy. We developed and optimized a targeted DIA LC-MS/MS MRM-HR method for quantifying site-specific occupancy at diverse N-glycosylation sequons in yeast cell wall glycoproteins and used this method to compare global site-specific glycosylation under two LLO stress conditions: deficiency of Alg6p or Alg7p, enzymes which catalyze distinct key steps in LLO biosynthesis. We found that a subset of N- glycosylation sites was differentially occupied in these different LLO stress conditions, consistent with active regulation of site-specific N-glycosylation depending on distinct amino acid sequence features surrounding the glycosylation sequons. Our results are consistent with a model in which cells under glycosylation stress maintain efficient glycosylation at critical sites in glycoproteins through regulated recognition of specific extended N-glycosylation sequons.

#42 - A modular post-production glycoengineering system using an Artificial Golgi Column

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Protein glycoengineering can be used to improve glycoprotein interactions and target therapeutic proteins. Traditional in vitro glycoengineering methods are costly, requiring lengthy incubation times of hours or days. Here, we show the promise of an artificial Golgi column (AGC) by performing in vitro glycan modification of a protein using a continuous flow immobilised enzyme reactor (IMER) with glycosyltransferases (GT), B4GalT1 and ST6Gal1, which add sugars (galactose and sialic acid respectively) to specific scaffold N-glycans present on targeted proteins. Using a liquid chromatography system, we injected unmodified protein, required substrate phosphate-sugars, and essential MnCl2 to the GT-IMERs, mimicking immobilised GT's within the trans-Golgi network. By adjusting the residence time of proteins to these GT-IMERS, we can modulate the completeness of enzymatic reaction, with total reaction completion taking just 4 minutes for the B4GalT1-AGC. Combining continuous flow and immobilised enzyme(s) increases interaction with substrates, decreases reaction times to minutes rather than hours, reduces the need for downstream enzyme removal, and enables repeat and sequential reactions. GT-IMERS are stable under continuous reaction conditions for up to 8 hours and can be stored for several weeks. By joining different GT-IMERS in sequence, multi-glycosyltransferase reactions become possible. This glycoengineering of proteins shows promise for future industrial applications with the potential for large-scale, "dial-up" glycan modification.

#43 - Developing a glycoproteomics analytics workflow for characterisation of recombinant proteins

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Recombinant proteins are essential for a wide range of applications such as biotherapeutics, diagnostics, enzymes, human and veterinary vaccines. In our facility, four distinct expression systems, namely *E. coli*, yeast, baculo-insect cell and mammalian cell, are used for producing recombinant proteins. Following protein purification, proteins are characterised using various technologies to determine key attributes such as identity and size. Besides these key attributes, post translational modifications such as glycosylation is shown to be critical for protein folding, stability and function [1]. Glycosylation is one of the most common protein modifications and is complex to characterise due to the diversity of the type and configuration of glycans attached to a recombinant protein. Variations in glycosylation patterns are known to be directly related to diseases like cancer, genetic and blood disorder [2]. Taking the importance, complexity and growing interest in protein glycosylation, there is an enduring need to develop analytical capabilities to characterise glycosylation patterns of proteins for biomedical research. To better understand how glycosylation differs and affects recombinant proteins expressed from yeast, baculo-insect cell and mammalian cell, several protein candidates produced from our facility were selected for glycoproteomic characterisation. We have identified a workflow to detect glycopeptides from recombinant proteins. To determine suitability of sample for mass spectrometry analysis, quality attributes of the recombinant protein sample are assessed. Samples are then denatured, reduced, alkylated, and proteolytic cleaved. Digested peptides are desalted and concentrated with a C18 column then analysed by LC-ESI-MS/MS (Prominence nanoLC system and TripleTof 5600). Data was processed using Byonic where we identified characteristic glycan types from recombinant protein candidates produced from the different expression systems.

#44 - Evaluating mucin O-glycans from faecal samples to study the association between gut microbiome and mucin glycosylation

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Mucins are the characteristic heavily O-glycosylated proteins present in the protective mucus layer that coats the inside of the digestive tract. The glycosylation state of mucins is dynamic and has been shown to be correlated with gut microbiome[1] and gut health[2]. Changes in general health have direct and indirect downstream effects on the microbiome of the digestive tract and the glycosylation of these mucins. Mucous collection and analysis from the colonic region, though informative, requires sacrificing the animal, and is not a feasible option in designing studies that seek to include non-laboratory animals. Mucin purification from faecal samples is a suitable alternative for sample collection, however, most existing mucin analyses from faecal samples do not employ sophisticated glycoanalytical approaches and only report on the total glycan content of the mucins. This study develops a method to isolate and purify high molecular weight mucins from mice faecal samples to characterise mucin O-glycans, compares it against the colonic mucin O-glycan profile, and identifies its correlation with a previously obtained corresponding dataset on the mouse colonic microbiome that binds to these glycan structures. These faecal and colonic mucin samples were collected from a mouse model system of colorectal cancer (C57BL/6 male mice chemically induced with azoxymethane and dextran sulphate to develop colorectal cancer (CRC)). This work combines mucin isolation methods and liquid chromatography-tandem mass spectrometry-based glycomics to create a reference library of CRC mouse (*Mus musculus*) model O-glycan structures on gut mucins isolated from their faeces. In particular, structural aspects of mucin sulphation, fucosylation and sialylation will be determined as these epitopes are known to be selective for binding to different microbial species in the gut. The method development will facilitate further research on changes in state of mucin glycosylation in animals in response to variable conditions through non-invasive strategies.

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2. 2. Yamada, T., et al., Mucin O-glycans facilitate symbiosynthesis to maintain gut immune homeostasis. 2019. 48: p. 513-525.

#45 - Comparative glycan profiling of breast cancer cell line derived small extracellular vesicles

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It is well recognized that the extracellular vesicles (EVs) constitute a promising biomarker target by transferring biomolecules to other neighboring cells. Triple negative breast cancer (TNBC) is a rare, basal-like and highly aggressive breast cancer subtype with poor prognosis. Due to its hormone-independent behavior, treatment methods currently available for hormone dependent breast cancers, such as the HER2 positive breast cancer, are ineffective. Hence, it is important to identify specific biomarkers for the early detection and accurate typing of the breast cancer. Numerous studies on proteins associated with EVs are available, however comprehensive glycomic characterization of EVs has been less explored. Therefore, we characterized the N-glycans and O-glycans on EV glycoproteins from 2 different cell lines: triple negative breast cancer (MDA-MB-231) and hormone-dependent (MCF-7).
EVs from both the cell lines were purified from the serum-free cell culture supernatants by differential ultracentrifugation and were later characterized for their size distribution and concentration using nanoparticle tracking analysis. From equal amounts of EV proteins from both cell lines, N-glycans were released enzymatically by PNGase F and reduced, and O-glycans were chemical released by reductive β-elimination. Glycans were desalted and resolved using porous graphitized carbon chromatography and analyzed by mass spectrometry. We were able to observe distinct differences between the N- and O-glycome of the EVs from the two cell lines. Interestingly, the glycome profile of EVs fraction from MDA-MB-231 and MCF-7 were different from the protein profiles of their secretome, previously published (1). As glycomics is associated with a wide range of diseases, knowledge of the profile of glycans may help in developing a novel platform technology for multiplexed glycan mapping for addressing the challenges in cancer biomarker discovery.

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#46 - Development of a new glycan-related pathway repository: GlycoPathwayRepo

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GlycoPathwayRepo is a repository developed to allow biologists to directly register glycan-related pathway information and obtain an accession number. The acquired information such as biochemical reactions, catalysis events, and resources including proteins, complexes, small molecules, enzymes, and glycans are transformed into a machine-readable format using Semantic Web Techniques including Resource Description Framework (RDF) and SPARQL Protocol and RDF Query Language (SPARQL). The entered resources are described with the Biological Pathway Exchange (BioPAX) ontology, which is a standard language to facilitate data sharing between pathway databases [1] and controlled vocabularies to connect the resources to data in other pathway databases or refer to the same data in primary biological databases. Users can easily check their pathway data after completing the input of their data on the via a visualization of the pathway. Our repository will allow users to inspect various biological commonalities, such as the reactions that are common in different pathways, the reactions of glycoenzymes that have common substrate specificities between different pathways, or the glycans that exist between disease and normal states in the same pathway. Tremendous amounts of glycome data have revealed that glycosylation across global cellular activity has biological importance to the

comprehensive understanding of cellular function [2]. We expect that our repository will contribute to accelerating data sharing of glycobiology with other omics data by providing tools where wet-lab researchers can share their meaningful data in a formalized and exchangeable formatd.

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2. "Glycosylation in Cellular Mechanisms of Health and Disease". Kazuaki Ohtsubo et al., 2006;126:855-867. Cell

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#47 - Analysis of glycans recognized by lectins using the MCAW multiple glycan alignment tool and experimental data of lectin-glycan interactions.

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The Consortium for Functional Glycomics (CFG) and Lectin Frontier DataBase (LfDB) are available on the web as databases of experimental data of interaction between glycans and lectins. CFG provides such data as a result of analyzing glycan binding proteins with glycan arrays, while LfDB provides data on the binding affinity between lectins and glycans based on frontal affinity chromatography.

Meanwhile, we have developed the Multiple Carbohydrate Alignment with Weights (MCAW) web tool using bioinformatics [1]. This tool can visualize the common glycan substructures among the inputted multiple glycan structures as a profile. Therefore, when the MCAW tool is executed with glycan structures showing binding affinity obtained to a particular glycan binding protein, the commonalities in the glycans can be seen. So far, the MCAW tool has been used to analyze the glycan array experimental data of CFG. As a result, we found various interesting patterns in which the glycan recognition patterns of lectins in the literature coincide with the profiles visualized from the MCAW tool [2]. In this presentation, we have further analyzed the affinity data of glycans and lectins in LfDB, where experiments are different from those in CFG, using the MCAW tool, and compared the results of glycan profiles obtained for the same lectins published in CFG and LfDB, respectively.

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2. [2]Hosoda, M. et al. Carbohydr. Res. 2018;464: 44-56.

#48 - Development of an efficient glycan structure search tool using a new score matrix based on monosaccharide structures

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We have been studying the methods to calculate similarity scores for pairs of glycans and to efficiently search for glycan structures in existing datasets, such as databases.

Because glycan structures can be formulated as tree structures, KCaM was developed as a tool for aligning the tree structures of glycans. The score obtained by the alignment can be used as a similarity score between the glycans.

Additionally, a score matrix[1] that represents the similarity of glycosidic linkage pairs (including the bound monosaccharides) was developed around the same time, based on existing glycan data sets at that time. However, this score matrix had not been applied very often because it was not versatile and ideally had to be re-created each time more glycan structures were found.

Therefore, we attempted to develop a more versatile score matrix for glycans that was independent of any glycan data set. . As a first attempt, we developed a monosaccharide substitution matrix that evaluated the similarity between monosaccharides by considering their physicochemical properties and their three-dimensional structures. As a result, we were able to create a novel monosaccharide substitution matrix[2].

This monosaccharide substitution matrix was applied to KCaM and validated by demonstrating the improved performance of KCaM by incorporating our score matrix and searching for glycans in the GlyTouCan repository.

This matrix is independent of any glycan database, and it incorporates the physicochemical properties of monosaccharides, making it the first of its kind.

The bioinformatics implications of being independent of any database are that the same matrix can be used for glycan comparisons for any species. Thus, we claim that the method for developing this matrix is universal.

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2. Akihiro Fujita, Aoki-Kinoshita, K.F., Development of a novel monosaccharide substitution matrix for improved comparison of glycan structures, Carbohydrate Research, 511: Online, 2022.

#49 - Glycoproteomics MS/MS data analysis benefits from glycomics-informed search strategies

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In our recent multi-institutional 1st HGI study[1], we explored search parameters of LC-MS/MS data affecting the glycopeptide identification process and found that the glycan search space is a critical variable to ensure comprehensive glycoproteomics data analysis. However, the exact impact of the glycan search space remains unexplored. Herein, we investigate if the *N*-glycopeptide data analysis benefits from our glycomics-assisted glycoproteomics approach, which defines the glycan search space using quantitative glycomics, as opposed to uninformed approaches using predefined glycan databases. Glycoproteomics LC-MS/MS data of human serum were systematically searched using the Byonic search engine, varying only the *N*-glycan search space parameter. Our informed approach employed a set of 41 *N*-glycan compositions observed in serum and subsets thereof, while the uninformed approaches used a condensed, moderate and extended glycan search space of common mammalian *N*-glycans predefined in Byonic chosen to simulate typical glycoproteomics data analyses without prior knowledge. The relative performance of the different search approaches was evaluated based on the glycoproteome coverage (sensitivity), glycopeptide accuracy (FDRs) and search time. Importantly, we found that the glycomics-informed search strategy using all 41 serum *N*-glycans provided substantial benefits over the three uninformed approaches by dramatically improving the accuracy of identified glycopeptides (lower FDR) and by reducing the search time (hours to minutes) without compromising the glycoproteome coverage. The relative performance of the informed approach was improved further in terms of higher accuracy and reduced search time when using smaller *N*-glycome subsets comprising only the most abundant serum *N*-glycans while still retaining a high glycoproteome coverage. In conclusion, our study documents the notable benefits of applying glycomics-assisted glycoproteomics to define the glycan search space ahead of the glycopeptide data analysis and confirms more broadly that knowledge-driven sample-specific search strategies are required to achieve high-performance glycoproteomics data analysis that remains a critical bottleneck in the field.

1. Kawahara, R. et al. Community evaluation of glycoproteomics informatics solutions reveals high-performance search strategies for serum glycopeptide analysis. Nat. Methods 18, 1304-1316, doi:10.1038/s41592-021-01309-x (2021).

#50 - Benchmarking the utility of open-source library-free DIA tools in the identification and quantification of glycan modifications using mass-spectrometry

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Data Independent Analysis (DIA) has been recently established as a robust and reproducible peptide quantification and identification technique in the field of proteomics, overcoming the limitations of Data Dependent Analysis (DDA). Recent advancements in library-free analysis methods streamline DIA workflows, bypassing the need for DDA library generation, which makes savings on time, resources, and sample volumes. This has made DIA a valuable analysis pathway for fields where DDA based library generation is not practical, however the utility of these tools for the identification of post-translational modifications (PTMs) has not been thoroughly established.

We aim to establish the utility of existing library-free DIA tools to the field of glycoproteomics and compare against DDA based glycan identification methods.

Using in-house generated proteomic/glycoproteomic data as well as datasets available from online repositories we assess the most-current versions of the open-source software; DIA-NN, DIA-Umpire (MaxQuant), OpenSWATH, Skyline, and FragPipe, and set a baseline for glycoform identification using the proprietary DDA software Byonic. We initially use a known glycoprotein mixture of IgG, lactalbumin, and fetuin, followed by vigorous testing across HeLa lysate, human serum, and human saliva. Each software is assessed on the basis of reproducibility, quantification linearity, and number of proteins and PTMs identified, with a particular focus on O- and N- linked glycosylation.We compare these key metrics against existing DDA workflows, summarise the relative performance of each tool, and discuss possible refinements to DIA workflows to maximise their utility for the field of glycoproteomics.

#51 - Evidence of endoplasmic reticulum stress in diabetic platelets

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Background:
Platelet hyperactivity in diabetes mellitus (DM) contributes to the increased cardiovascular risk and decreased efficacy of antiplatelet agents in this group of patients. Endoplasmic reticulum (ER) stress contributes to endothelial dysfunction in patients with DM, but whether the ER stress response also contributes to platelet hyperactivity is not well-studied. Tunicamycin is an N-glycosylation inhibitor that has been shown to induce ER stress in vitro and in vivo, but its effect on platelets remains unclear.

Aim:
We aimed to identify changes in intracellular and released platelet proteins in patients with and without diabetes who have known or at risk of cardiovascular disease. We aimed to recapitulate the identified ER stress pathway seen in the diabetic platelet in vitro using the N-glycosylation inhibitor tunicamycin.

Method:
A total of 76 patients (DM = 42, non-DM = 34) were recruited. Platelets were isolated and separated into lysate and releasate, with or without stimulation with 0.025U/mL thrombin. Platelet activation was assessed by mobilisation of CD62p and PAC1. Proteins were identified by LC-MS/MS and Western blot. Isolated platelets from healthy donors were treated with tunicamycin (5µg/mL) before platelet activation was quantified by flow cytometry and ER stress pathway induction by Western blot.

Results:

There was a significant and positive correlation between serum fructosamine, a marker of glycaemic control, and SEC61B, a part of a multi-functional ER translocon complex. DM platelets had significantly increased phosphorylation of IRE1, a major signalling pathway in the ER stress response. Tunicamycin did not appear to significantly increase CD62p mobilisation or PAC1 positivity, although there appeared to be activation of platelet ER stress pathways.

Conclusion:

We have identified a platelet ER stress response in patients with DM, which may contribute to platelet hyperactivity. Further studies are required to identify a pharmacologic treatment to recreate the DM effect on platelets in vitro.

#52 - The timsTOF fleX: A New Age of *N*-Glycan MALDI-MSI

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Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry imaging (MSI) is a robust and rapid technique which allows the spatial mapping and visualisation of analytes, such as intact proteins, tryptic peptides, lipids, metabolites and *N*-glycans, across fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) tissue sections (1). With the recent release of Bruker Daltonic's timsTOF fleX MS system, MALDI-MSI has moved into a new age of spatial omics, with the benefit of higher sensitivity and faster acquisition rate relative to older generation instruments on the market (2). Our group has further developed and refined our existing *N*-glycan MALDI-MSI workflows to increase the number of *N*-glycan compositions identified from various types of FFPE tissue, including articular cartilage and subchondral bone from knee osteoarthritis patients, lungs from mice with and without mucopolysaccharidosis type I and III, and ovarian, endometrial, cervical and vulvar cancers from early- and late-stage patients. Furthermore, our group has established an *in-situ* tandem MS (MS/MS) fragmentation protocol which can be implemented post-MALDI-MSI analysis to structurally characterise and confirm putative *N*-glycan masses of interest. Lastly, our group has successfully separated structural isomers (i.e., glycoforms) *in-situ* using trapped ion mobility mass spectrometry (TIMS), following *N*-glycan MALDI-MSI analysis, on the same tissue section. With these complimentary methods in mind, a wealth of information can be acquired from a single tissue section, thereby reducing the sample preparation time previously required while increasing the number of *N*-glycan compositions and structural isomers that can be spatially mapped.

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#53 - The role of CD44 isoforms and glycoforms in endothelial adhesion of human tumor cells

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Most cancer-related deaths are due to metastatic spread of primary tumors. One crucial step in hematogenous metastasis is the dynamic adhesion of circulating tumor cells to the endothelial cells of the vessel walls, which initiates extravasation of tumor cells to distant sites. This adhesion is mediated by interactions of adhesion molecules on tumor cells and their binding partners on endothelial cells. A much noticed but obscure adhesion molecule in this context is CD44. Due to its numerous isoforms and glycoforms, our understanding of the role of CD44 in metastasis remains elusive and needs to be improved by further research.

In the first step of the present study, we established a stable and efficient shRNA-mediated CD44 knockdown (of all isoforms) in the two human tumor cell lines HT29 and MeWo, which resulted in less dynamic endothelial adhesion in case of MeWo, but not HT29 cells. Paired-end RNA sequencing revealed a predominant expression of CD44 isoform 3 in HT29 and of isoform 4 in MeWo cells, which was confirmed by isoform-specific qRT-PCR and by exon-specific antibodies used for flow cytometric tumor cell analyses. Accordingly, Western Blot analyses showed a higher molecular weight of CD44 in HT29 (140 kDa) in comparison to MeWo cells (85 kDa). Chemical inhibition of *O*-GalNac glycosylation and enzymatic cleavage of *N*-glycans and terminal sialic acid residues indicated the presence of *O*- and *N*-glycans with respective sialylation on CD44 on both cell lines, but to different extents. Preliminary mass spectrometric analysis of proteins co-immunoprecipitated with anti-CD44 identified proteins of the cytoskeleton (vimentin, actin, α/β tubulin), which were downregulated in the CD44 knockdown cells of MeWo but not HT29. Further analyses with additional cell lines are currently ongoing and will show whether a certain CD44 isoform and glycoform pattern emerges in the context of tumor cell adhesion to endothelial cells.