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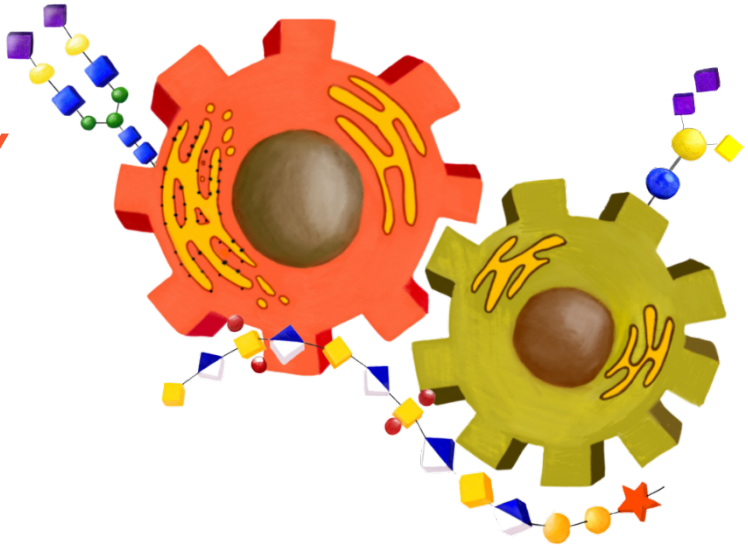

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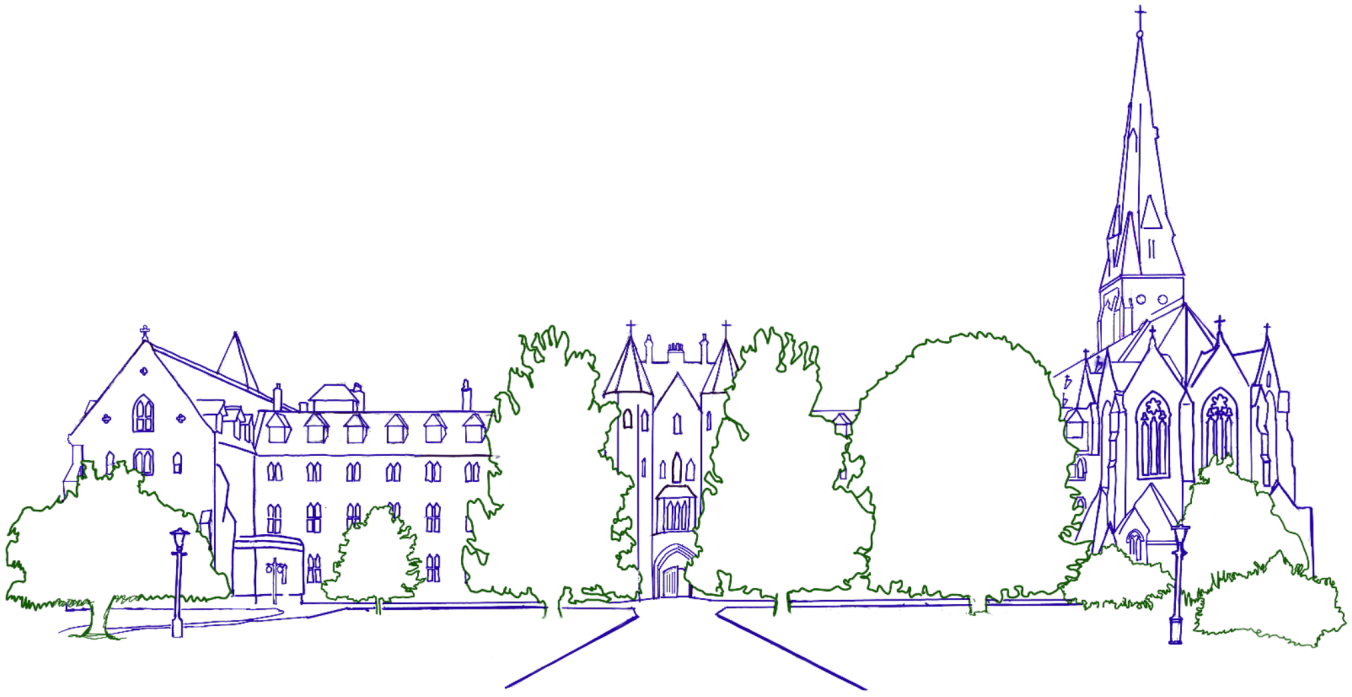

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Selected Talks

(SLT1) Site-specific reprogramming of the tumour-immune microenvironment through sialic acids

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Malignant transformation is accompanied by severe changes in glycosylation, resulting amongst others in elevated expression of sialylated structures. This hypersialylation can initiate immune evasion via the interaction with inhibitory Siglec receptors on immune cells. However, it is currently unknown whether the impact of sialylation on anti-tumour immunity is influenced by the tumour location.

Transcriptomic analysis of human colorectal tumours indicated that the sialic acid-Siglec axis is elevated in peritoneal metastasis versus primary or liver tumours. Next, we employed CRISPR/Cas9 to knockout the *CMAS* gene in the murine colorectal cancer cell line CT26, thus creating variants devoid of cell surface sialic acids. We challenged mice with our glycovariants and observed a strong reduction in tumour growth of the CT26-CMAS KO cells both in a subcutaneous as well as in a peritoneal metastasis model. This difference was absent in immunodeficient mice, signifying an immune-dependent effect. Depletion of $CD8^+$ T cells did not affect tumour growth of subcutaneous CMAS KO tumours, suggesting a role for other immune cell subsets.

Indeed, multicolour spectral flow cytometric analysis revealed a different maturation pattern and less exhausted phenotype of NK cells and an increased influx of γδ T cells. In contrast, peritoneal CMAS KO tumours harboured elevated levels of B cells, $CD4^+$ T cells and activated NK cells, while γδ T cell numbers were lower.

In conclusion, we show that the immune evasion installed by sialic acids is highly dependent on the local tissue in which the tumour resides. We are currently exploring whether inhibition of sialic acid synthesis could be an effective treatment strategy to counteract CRC outgrowth.

(SLT2) ER *O*-glycosylation in synovial fibroblasts drives cartilage degradation

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How arthritic synovial fibroblasts (SFs) activate cartilage ECM degradation remains unclear. GALNT enzymes initiate O-glycosylation in the Golgi; when relocated to the ER, their activity stimulates ECM degradation.

We found that in human rheumatoid and osteoarthritic synovial SFs, GALNTs are relocated to the ER. In an RA mouse model, GALNTs relocation occurs shortly before arthritis symptoms and abates as the animal recovers. An ER GALNTs inhibitor prevents cartilage ECM degradation in vitro and expression of this chimeric protein in SFs results in the protection of cartilage. One of the ER targets of GALNTs is the resident protein Calnexin, which is exported to the cell surface of arthritic SFs. Calnexin participates in matrix degradation by reducing ECM disulfide bonds. Anti-Calnexin antibodies block ECM degradation and protect animals from RA. In sum, ER O-glycosylation is a key switch for the activation of arthritic SFs.

Glycosylated surface Calnexin could be a therapeutic target for arthritic conditions[1].

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(SLT3) *N*-glycosylation engineering in chimeric antigen receptor T cells enhances anti-tumour activity

Nele Festjens[1,2,4], Elien De Bousser[1,2,4], Evelyn Plets[1,2], Laura Vuylsteke[1,2], Leander Meuris[1,2,4], Annelies Van Hecke[1,2], Elise Wyseure[1,2], Marie Goossens[1,2], Laure De Pryck[1,2,4], Stijn De Munter[3,4], Bart Vandekerckhove[3,4], Nico Callewaert[1,2,4]

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Recently, chimeric antigen receptor (CAR) T cell technology has revolutionized cancer immunotherapy. This strategy uses synthetic CARs to redirect T cells to specific antigens expressed on the surface of tumour cells. Despite impressive progress in the treatment of haematological malignancies with CAR T cells, scientific challenges still remain for use of CAR T cell therapy to treat solid tumours. This is mainly due to the hostile tumour microenvironment and CAR-related toxicities.

As the glycans decorating the T cell surface are implicated in T cell activation, differentiation, proliferation and in the interaction of human T cells with tumour cells, we are studying the influence of CAR-T cell glycosylation on their efficacy, by systematically deconstructing parts of their glycome. Here, we zoom in on findings obtained by reducing the density of N-linked poly-LacNAc modifications, which synthesized onto the β1,6-GlcNAc branch introduced by N-acetylglucosaminyltransferase V (GnTV, encoded by *Mgat5*).

We discovered that MGAT5 knockout (KO) through CRISPR-Cas9 engineering of anti-CD70 nanoCAR T cells results in the robust elimination of highly immunosuppressive tumours in mice. Additionally, in mice that had been cured of their primary tumour following such MGAT5 KO CAR T treatment, we also observed enhanced clearance many months later of a relapse-mimicking tumour, and we detected consistently higher numbers of patrolling CAR T cells in circulation, altogether indicating an enhancement of functional immunological anti-tumour memory[1].

These exciting results demonstrate long-term functional persistence of these glyco-engineered CAR T cells upon clearance of solid tumours, which is the most sought-after enhancement in CAR T therapy. Additionally, we show that knocking out *Mgat5* in CAR T cells targeting CD20 has the same beneficial effect on tumour control and CAR T cell persistence in the highly aggressive Raji B-cell lymphoma model. This is a very difficult to achieve result with any other known CAR T manipulation, providing strong proof of concept that glycocalyx engineering of CAR T cells could be of general benefit to combat cancer.

We are presently elucidating the molecular mechanisms underlying this promising CAR-T therapeutic enhancement, by single cell analysis of tumour-infiltrating CAR-T cells and by building an *in vitro* model for the observed enhanced functional persistence in the face of chronic tumour cell exposure.

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(SLT4) Unravelling the role of extended *O*-glycans in human biology: Insight from the pathogenic variants of *C1GALT1C1*

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Cosmc, encoded by the X-linked *C1GALT1C1*, is a specific chaperone in the endoplasmic reticulum for T-synthase, which becomes a resident Golgi enzyme. T-synthase is the only enzyme responsible for adding galactose to the Tn antigen (CD175) (GalNAcα1-S/T/Y) to generate T-antigen (Galβ3GalNAcα1-S/T/Y). This T-antigen serves as a common precursor for thousands of different extended *O*-glycans, which are important in many cellular functions, including cell signalling/adhesion, cell-cell communications, interactions with the microbiome, etc. As such, protein O-glycosylation plays a critical role in human health and diseases. However, the impact of *C1GALT1C1* mutation in humans is largely elusive.

This study seeks to address the knowledge gap regarding the impact of *C1GALT1C1* mutations on human health by investigating two distinct pathogenic variants in *C1GALT1C1:* 1. a germline loss of function hemizygous variant c.59C>A (p.Ala20Asp; A20D-Cosmc), and 2. a *de novo* mosaic variant (c.202C>T, p.Arg68*; Cosmc-R68) found in a female patient. A20D-Cosmc patients display a diverse spectrum of symptoms, giving rise to C1GALT1C1-CDG[1]; the symptoms include developmental delay, short stature, intellectual disability, and acute kidney injury (AKI). Mechanistically, A20D-Cosmc patients have a remarkable reduction of both Cosmc and its mRNA expression in a cell or tissue-specific manner, consequently impacting T-synthase activity resulting in varying levels of pathological Tn antigen expression. We found that multiple quality control pathways that regulate the dramatic loss of A20D-Cosmc.

On the other hand, considering the Cosmc-R68 patient, a 5-year-old girl was born prematurely at 33 weeks of gestation. At 31 weeks of gestation, she presented with non-immune hydrops fetalis, which later resolved. In infancy, she exhibited axial hypotonia and global developmental delays, particularly speech. At 10 months of age, proteinuria was detected and has remained persistent. She carries the variant as a mosaic in buccal cells (allelic fraction 27%). Mechanistically, we found the mutant Cosmc protein in the patient's leukocytes is truncated and nonfunctional, leading to reduced T-synthase activity; in our analysis of the patient's red blood cells (RBCs), leukocytes, and serum compared to healthy controls, we observed significantly reduced expression of normal O-glycans and a concomitant increased expression of the Tn antigen, consistent with the reduction of T-synthase activity.

The Cosmc-R68 patient is the first example of a true mosaic loss-of-function variant in *C1GALT1C1* in a female, which occurred post-zygotically during embryogenesis. Collectively, this body of work represents the first description of patients with unique *O*-glycosylation defects caused by pathogenic variants in *C1GALT1C1*, presenting a diverse spectrum of disorders.

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(SLT5) Galactosylated and afucosylated glycoforms of intravenous immunoglobulin control inflammation

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The mechanism of action of intravenous immunoglobulin (IVIG) as an immunomodulatory agent remains uncertain[1,2]. As afucosylated IgG predominantly binds to Fcγ receptor IIIa (FcγRIIIa) on circulating natural killer cells in healthy individuals[3], we hypothesized that afucosylated IVIG might serve as an antagonist for FcγRIIIa and modulate immune responses.

Here, we generated fucosylated or afucosylated glycoforms of normal polyclonal IgG bearing sialylated, galactosylated or agalactosylated Fc glycans by chemoenzymatic glycoengineering to investigate whether the IgG glycoforms could inhibit antibody-dependent cellular cytotoxicity (ADCC). Importantly, galactosylated and afucosylated [$(G2)_2$] IgG bound FcγRIIIa and inhibited ADCC most potently. In addition, the $(G2)_2$ glycoforms of IVIG showed stronger anti-inflammatory activity than a 10-fold higher dose of native IVIG in mice with collagen antibody-induced arthritis.

These findings demonstrate that the anti-inflammatory activity of IVIG is in part mediated via blockade of activating FcγRs by galactosylated and afucosylated IgG glycoforms(s). This study suggests the therapeutic potential of glycoengineered IVIG and Fc as alternatives to native IVIG[4,5].

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(SLT6) Glycomics microarrays for profiling host immune-pathogen interactions and measuring adaptive immune response

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the global coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV-2 infection typically stimulates production of serum antibodies against various viral antigens (Ag) over time.

We previously showed a significant association between disease severity and serum antibody isotype binding intensity for specific viral protein antigens including spike protein fragment S1, which depended on antigen glycosylation[1]. Serum antibody level is also associated with neutralisation and clearance as Fc receptors (FcR) and effector functions are engaged. However, antibody-Ag immune complex receptor binding has not yet been associated with COVID-19 disease severity and phagocytosis. In addition, the influence of Ag glycosylation on immune complex receptor interactions is unclear.

Serum IgG core fucosylation was revealed in the severe COVID-19 disease cohort by lectin microarray profiling and HPLC analysis, which was absent in the mild and moderate disease and pre-pandemic healthy donor cohorts. Multiplexed measurements demonstrated that the affinity (Kd) of IgG from severe patients for the S1 Ag was highest by 3 weeks post-infection, and decreased over time while S1 K_D of mild and moderate disease patient IgG increased. Serum IgG from the different disease severity and healthy cohorts were also complexed with various viral protein Ags and incubated with Fc receptors (FcR) and pattern recognition receptors (PRR) in a custom microarray format. Serum IgG-Ag immune complexes modulated FcR interactions and Kd compared to Ag alone, and Ag glycosylation also contributed to FcR and PRR binding. Phagocytosis of bead-bound S1 Ag was assessed in the presence and absence of serum IgG from the different cohorts. Overall, higher binding to CD64 and greater phagocytosis was associated with severe disease serum IgG and fucosylation.

Further, custom glycomics microarray platforms provide a novel and rapid methodology to identify and measure immune interactions, which can contribute to disease severity prognostics and treatment modality decisions.

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(SLT7) Detection of immune cell glycosylation as an indicator of metabolic activity in the tumour tissue microenvironment using *N*-glycan and multiplexed-IHC mass spectrometry imaging

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Our group has developed multiple N-glycan targeted mass spectrometry imaging workflows that have been applied to detect the spatial distribution of *N*-glycans in tumour, stroma and adjacent normal cellular regions in thousands of clinical cancer tissues. Until recently, after improvements in instrumentation and new multiplexed MALDI-IHC assays, mapping the glycosylation of tertiary lymphoid structures and related immune cell clusters in these tumour tissues has not been emphasized.

Generally, immune cells express numerous glycan binding receptors such as siglecs, C-type lectins, galectins and selectins, which recognize alterations in tumour cell surface glycosylation and either enhance immunostimulatory or immunoinhibitory pathways. We hypothesize that metabolically active immune cell clusters within solid tumour tissues will exhibit more intense *N*-glycan signal than immune suppressed clusters.

Utilizing 71 tissue samples collected from patients with varying stages of prostate (51), pancreatic (12), colon (6) and kidney (2) cancers, the *N*-linked glycan profiles of >300 immune cell clusters within and surrounding these tumours was determined using Matrix Assisted Laser Desorption/Ionization-Mass Spectrometry Imaging (MALDI-MSI). Additionally, we analysed the cell composition of a subset of immune clusters with photocleavable mass tags from AmberGen using MALDI-MS and highly multiplexed immunohistochemistry (MALDI-IHC).

We selected antibodies targeting immune cell markers (CD3, CD8, CD11b, CD11b, CD20, CD44 and CD68) and extracellular matrix components (E cadherin, Collagen 1A1, and smooth muscle actin) for this analysis. We found that high mannose and a diverse set of core fucosylated N-linked glycans are typical of immune cell clusters within and surrounding pancreas, colon and prostate tumours. Additionally, N-glycan signal intensity was lower within intra and peri tumoral immune cell clusters versus immune cell clusters more distal from the primary tumour. Further, we established that CD20+ B cells account for the majority of signal colocalized with these immune cell clusters with additional signal from CD3+ T cells, consistent with tertiary lymphoid structures. Prostate and pancreatic cancers are currently two of the least responsive tumours to check-point inhibitor immunotherapies. A majority of the immune clusters in these two tumour tissue types lacked detectable *N*-glycan signal, while detection in colon and kidney was more prevalent.

The significant role of glycosylation in modulating activity within the tumour immune microenvironment suggests that identifying tumours with robust immune cell glycosylation signals could better identify patients that are more likely to respond to immunotherapy treatment. Ongoing studies are evaluating the link between circulating and tissue immune cell glycan profiles, progressive stages of these “immune hot or cold” cancers, and immunotherapy response.

Speed Talks

(SPT1) Potential Sialic Acid and Glycomic Biomarkers of Cardiovascular Disease

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Cardiovascular disease (CVD) is characterized by high levels of inflammation which has been associated with elevated concentrations of *N*-acetyl neuraminic acid (Neu5Ac) in plasma. 9-*O*-Acetyl-*N*-acetyl-neuraminic acid (Neu5,9Ac$_2$), has also been of interest as a biomarker for diseases such as breast cancer but has not yet been studied in the case of CVD.

Neu5Ac and Neu5,9Ac$_2$ concentrations were determined by quantitative analysis using liquid chromatography in plasma samples obtained from both patients with advanced CVD and healthy controls by DMB labelling of sialic acids. Thirty patients were selected for each cohort; samples were sourced from a biobank. Mean concentrations of Neu5Ac and Neu5,9Ac$_2$ were significantly elevated in the patient group (Neu5Ac: $P < 0.001$; Neu5,9Ac2: $P < 0.04$). Receiver operator curve analysis (ROC) further revealed the predictive power (AUC) of the two markers (Neu5Ac: 0.86; Neu5,9Ac2: 0.71) indicating Neu5Ac may be of interest for distinguishing CVD patients from healthy controls. Further to this, a combined Neu5Ac/Neu5,9Ac$_2$ marker exhibited an improved AUC of 0.93. The sensitivity and specificity of each marker was then assessed, with the combined marker performing best overall showing low rates of both false negatives and false positives.

Overall, Neu5Ac may be a a good marker for CVD as expected from previous literature, addition of Neu5,9Ac$_2$ greatly improves the power to distinguish between disease patients and healthy controls.

Further analysis was undertaken to determine the *N*-glycan profile of each sample. ROC analysis was performed, and AUC values were compared with the data for Neu5Ac and Neu5,9Ac$_2$. Five *N*-glycans were significantly elevated in CVD patients with the species mainly being sialylated which may explain the elevation of sialic acid concentrations in the plasma of CVD patients. C-reactive protein was measured for each sample to act as a comparison to the markers in this study but performed extremely poorly (AUC: 0.50). The samples were also analysed via nanoparticle prefractionation (*U.K. Patent Application Number 2117557.5*). This allowed for the extraction of fibrinogen, a known inflammatory marker, and analysis of fibrinogen derived glycans.

(SPT2) IVD regeneration in a canine nucleus pulposus injury model – using sialylation inhibitor-loaded injectable hydrogels for IVD repair

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Intervertebral disc (IVD) degeneration is the leading cause of low back pain, affecting at least 619 million people globally in 2020[1]. IVD degeneration is characterised by tissue remodelling, resulting in loss of disc height and water content, and infiltration of blood vessels and nerve fibres, which are associated with increased pain sensation[2]. Similar to humans, dogs suffer from low back pain due to IVD degeneration and are diagnosed and treated in veterinary clinics. The clinical presentation, macroscopic and microscopic appearance, diagnostics, and treatment of IVD degeneration are similar in dogs and humans[3-5].

Glycans play vital roles in protein folding, cellular signalling, inflammation, and cell survival, making them attractive potential therapeutic targets[6]. Sialylation is a terminal modification of glycans and plays a pivotal role in many physiological and pathological processes[7]. In human IVD degeneration, hypersialylation is associated with tissue degeneration[8]. 3F-peracetyl-Neu5Ac, a global glycosylation inhibitor, is converted to the donor substrate analogue of CMP-Neu5Ac and inhibits sialyltransferase activity[9].

This study aimed to investigate the efficacy of a hyaluronic acid (HA) hydrogel, releasing 3F-peracetyl-Neu5Ac, as a regenerative therapy in a canine model of IVD degeneration over a prolonged course and assess the downstream effects of sialylation inhibition. The study hypothesised that an HA-based hydrogel could effectively inhibit hypersialylation in a model of canine IVD degeneration and improve the regenerative response to an HA hydrogel in IVD degeneration. All animal experiments were performed with ethical approval from the Research Ethics Committee of the Tokai University Hospital, Japan. Ten female beagles were subjected to five experimental conditions at eight spinal levels. Disc degeneration was induced by aspiration of nucleus pulposus tissue under four experimental conditions and allowed to progress for one month; the final group was approached but not invaded. Three experimental groups were administered HA-based hydrogel injections: one unloaded, one loaded with a low-dose inhibitor, and one loaded with a high-dose inhibitor; the final experimental group was administered saline infusion.

Before animal sacrifice, IVDs were MRI imaged on a Phillips Ingenia Ambition 3-Tesla MRI instrument, and high-quality sagittal T1, T2W, and T2 maps were recorded. IVDs were collected 112 days post-induction of degeneration, embedded in paraffin, sectioned into 5µm slices and fixed onto microscopic slides. Six samples per group were selected for matrix-assisted laser desorption/ionisation–mass spectrometry imaging (MALDI-MSI) N-glycan analysis. Tissue sections were deparaffinised, sialic acids stabilised and digested with PNGase-F enzyme before being coated in an α-cyano-4-hydroxycinnamic acid matrix by spray deposition using an HTX TM-Sprayer™. Tissue sections were analysed using a Bruker scimaX® instrument in the mass window of 650 – 3500 m/z, with a resolving power of 200.000 at m/z 933.31.

MRI showed improved disc height and increased disc water content in groups treated with the 3F-peracetyl-Neu5Ac loaded hydrogel compared to the hydrogel and saline-injected groups. MALDI-MSI analysis revealed a distinct and reproducible N-glycan expression in each experimental group. Inhibition of sialylation by 3F-peracetyl-Neu5Ac yields an expressed N-glycome that resembles untreated control tissues, including a reduction in expressed sialylated N-glycan species and an

increased expression of tetra-antennary N-glycans compared with the unloaded hydrogel and negative control tissue. Furthermore, a dose-dependent response was observed. MRI highlighted the impact of sialyltransferase inhibition by 3F-peracetyl-Neu5A on disc physiology, improving disc height and water content in 3F-peracetyl-Neu5Ac-loaded hydrogel-treated groups. Furthermore, this study successfully demonstrated the use of a global sialylation inhibitor to enhance the regenerative response of IVD tissues to an HA hydrogel in a preclinical canine model of IVD degeneration.

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(SPT3) Assessing glycosylation enzyme expression in activated innate lymphocyte subpopulations

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Innate and innate-like lymphocyte cells are emerging as important adoptive cell-based anti-cancer therapeutics due to their potent cytotoxic capacity. Importantly, innate lymphocytes, such as natural killer (NK) cells, and innate-like lymphocytes, such as mucosal-associated invariant T (MAIT) cells, are not restricted by major histocompatibility (MHC) complexes. While it is well recognised that activation of both NK cells and MAIT cells drive key metabolic changes required for functional activation, the impact of these activation-induced changes on glycosylation is unexplored. Research in other immune cell types have found differences in cell surface glycosylation, which in turn influence effector functions such as phagocytosis, cytokine production and antibody-dependent cellular cytotoxicity. Currently we lack knowledge on the regulation of glycosylation enzymes during and after activation of innate lymphocyte cell populations and we hypothesise that activation-induced changes in cellular glycosylation impact the anti-tumour functions of both NK cells and MAIT cells.

Next generation RNA sequencing datasets of stimulated human NK cells and human MAIT cells were identified and selected for suitability from the genome expression omnibus (GEO) data repository. Utilising the Galaxy EU web platform fastq files were aligned to the Human Reference Genome hg38 by HISAT2. Read counts were determined by htseq-count in reference to hg38 annotated features. Differential gene expression was examined using the DESEQ2 tool. From the data, read counts of glycan biosynthetic enzyme transcripts were collated and statistically analysed.

We identified ten suitable datasets relating to human MAIT cell activation and seven suitable datasets relating to human NK cell activation. The activation status of these datasets was confirmed through the upregulation of known genes associated with lymphocyte activation. We then analysed 126 gene transcripts encoding glycan biosynthetic enzymes. Both the activated MAIT cell and NK cell datasets had an upregulation of the transcripts encoding enzymes involved in the synthesis of the core glycans. This may be a reflection of the increased demand for protein production upon activation. In parallel there was a distinct upregulation of transcripts encoding enzymes responsible for the construction of polylactosamine chains (LacNAc), and a downregulation of the transcripts encoding fucosyltransferase activity.

This data provides an initial overview of the activation-induced changes in glycan biosynthetic enzyme transcripts in human NK cells and MAIT cells. These transcriptional changes can be utilised to predict protein-level changes, as well as downstream changes in cell surface glycans. This information is required to engineer cell surface glycans in the future, with a view to enhancing the anti-cancer properties of adoptive cell-based therapeutics.

(SPT4) Obesity affects the adipocyte glycosylation machinery and *N*-glycan profiles in mice

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Background: Obesity is a primary risk factor for type 2 diabetes (T2D), contributing to induce adipose tissue (AT) inflammation and insulin resistance[1-3]. AT inflammation results from the crosstalk between hypertrophic adipocytes and AT-resident immune cells, notably macrophages, which is partly driven by adipocyte-derived cytokines, metabolites and/or extracellular vesicles[4]. Glycans, which are complex carbohydrate structures attached to cell-surface or secreted proteins and lipids, could also play a role in such crosstalk, although very few data are currently available[5]. In this study, we investigated the impact of obesity on the adipocyte glycosylation machinery and cell *N*-glycan profiles in mice.

Method: C57BL6/J male wild-type mice were fed with a low-fat diet (LFD, 45% of energy derived from fat) or high-fat diet (HFD, 45% of energy derived from fat) for 15 weeks. Body weight, whole-body insulin sensitivity and epididymal white adipose tissue (eWAT) immune cell composition were determined. Adipocytes were isolated from eWAT, and subjected to N-glycomics and transcriptomics analyses, using MALDI mass spectrometry and bulk RNA sequencing, respectively.

Results: HFD increased body weight, adipocyte size and eWAT pro-inflammatory macrophages, and decreased insulin sensitivity, indicating whole-body insulin resistance and AT inflammation in obese mice. The *N*-glycomics analyses revealed distinct alterations of various types of *N*-glycans in adipocytes from obese mice, with increased bisecting GlcNAc structures in both hybrid (+174%; $p<0.05$) glycans and complex (+49%; $p<0.05$) di-antennary *N*-glycans. Adipocytes from obese mice also showed a higher abundance of tri-antennary and tetri-antennary *N*-glycan complex structures (+32%; $p<0.05$). Interestingly, the adipocyte expression of many genes related to the glycosylation machinery are significantly affected by HFD (16 upregulated, 14 downregulated; $-0.5<\log2FC>0.5$, $p<0.05$), including *Mgat4a*, a glycosyltransferase involved in the formation of tri-antennary branching structures, that was upregulated in adipocytes from obese mice.

Conclusion: The observed alterations in adipocyte glycan profiles in obese mice suggest that some specific carbohydrate structures may play a role in cell-cell interaction. Future studies are required to explore the functional implications of these glycan changes on macrophage activation and AT inflammation.

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(SPT5) Defining ER-localised *O*-glycosylation in pancreatic ductal adenocarcinoma

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Aberrant glycosylation is a hallmark of tumour progression and metastasis; however, its functional importance is still poorly understood. One mechanism driving changes in *O*-glycosylation is the GalNAc transferase activation (GALA) pathway, where *O*-GalNAc glycosyltransferases (GALNTs) relocate from the Golgi to the endoplasmic reticulum (ER)[1]. This pathway uniquely drives the glycosylation of ER-resident proteins. GALA is active in several solid tumours, including breast and liver cancers, and contributes to ECM degradation, tumour growth, and invasion[2–4]. This study investigates the role of GALA in pancreatic ductal adenocarcinoma (PDAC), focusing on tumour growth and the potential to define a GALA-driven glyco-signature for disease detection.

To explore GALA activity in PDAC and define its *O*-glycosylation targets, pancreatic tumour tissues and cell line models were analysed. Human tumour microarrays were stained with Vicia villosa lectin (VVL) and Helix pomatia lectin (HPL) to detect the Tn antigen, a hallmark of GALA activation. Genetic tools were used to modulate *O*-glycosylation and GALA activity, including cells with ER-localised GALNT1 (ER-G1), GALNT1-overexpressing cells (WT-G1), wild-type cells (WT), and GALA-inhibited cells (ER-2Lec). Tn levels were assessed in these models. Orthotopic injections of KPC47 cells expressing GALA constructs were performed to evaluate tumour growth and metastasis. Glycoproteomic profiling using high-throughput jacalin-agarose lectin weak affinity chromatography (sLWAC) followed by data-independent acquisition (DIA)[5] mass spectrometry identified glycopeptides with T and Tn glycans. Additionally, 18 patient-derived xenografts (PDXs) from the PaCaOmics cohort were analysed to validate findings in a clinically relevant context.

Pancreatic tumour tissues showed elevated Tn levels with an ER-like distribution compared to normal tissue, indicating GALA activation. Tn expression patterns revealed localisation primarily in CK19-positive tumour epithelial cells, though some patients exhibited high stromal Tn expression, suggesting tumour microenvironment heterogeneity. GALNT2 colocalised with the ER marker GRP78 in tumour tissues, and the ER protein calnexin was *O*-glycosylated, confirming GALA activity in PDAC. ER-localised GALNT1 (ER-G1) increased Tn levels compared to GALNT1 overexpression alone (WT-G1), indicating that ER localisation, not expression level, drives hyperglycosylation. GALA promoted ECM degradation in vitro, and ER-G1-expressing cancer cells enhanced tumour growth and metastasis in orthotopic models, while tumours failed to establish with GALA-inhibited cells (ER-2Lec). These findings suggest that GALA-induced hyperglycosylation impacts tumour growth regulation.

Glycoproteome profiling of ER-G1, WT-G1, and WT cell lines identified a distinct glyco-signature driven by ER-localised GALNT1. Hyperglycosylation targeted ER-resident proteins, ECM components, and cell membrane proteins. GALA activation increased Tn glycopeptides and poly-T and poly-Tn structures, indicating clustered *O*-glycosylation in the ER. These findings were validated in PDX PDAC tumours, which also exhibited an ER-like glyco-signature.

This study highlights GALA's role in driving a unique glyco-signature in pancreatic cancer. Hyperglycosylation targets ER-resident, extracellular, and cell membrane proteins, contributing to ECM degradation and tumour progression. Additionally, cleaved hyperglycosylated ECM or cell membrane proteins could be detected in serum, serving as potential biomarkers for PDAC detection. Future work will assess the cancer specificity of this glyco-signature and evaluate its diagnostic utility in serum from healthy individuals versus PDAC patients.

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(SPT6) (IR)Rational Design of Mucin Glycopeptides as Tumour Immunotherapeutic Agents

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Cellular glycans are critically important for an array of biological functions, in particular, as mediators of the mammalian immune response. During the development of tumours, the structure and presentation of these sugar chains are dramatically altered, leading to modified properties and often enhanced aggression of these neoplastic cells. One of the many varieties of glycans are O-linked chains attached to serine and threonine residues on proteins called mucins (aptly called, “mucin-type glycosylation”).

We have been exploring glycopeptides from the tandem repeat (TR) region of MUC4 (a mucin overexpressed on pancreatic ductal adenocarcinomas (PDAC) but absent in normal pancreas tissues) as immunogens for vaccines against PDACs. We have developed nanoparticle platforms that can deliver various TR glycopeptides designed in our lab to antigen presenting cells. This work led us to develop monoclonal antibodies (mAbs) that are specific for PDAC cells and we have shown that these mAbs accumulated selectively in PDAC tumours in vivo.

This presentation will outline the foundational studies related to this work and describe our latest vaccine/immunogen designs. Details on some newer mAbs we have developed, along with several therapeutic modalities (Antibody-Drug Conjugates (ADCs) and Chimeric Antigen Receptor-T cells (CAR-T cells)) that we are pursuing against pancreatic cancer will be discussed.

(SPT7) Deciphering Siglec ligands in cancer to improve immunotherapy

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Aberrant sialylation is a hallmark of cancer, contributing to immune evasion, metastasis and drug resistance[1]. Siglecs are a family of fifteen carbohydrate-binding proteins broadly expressed on the surface of immune cells[2-4] and have recently emerged as glyco-immune checkpoints as their sialylated ligands are upregulated on cancer cell surfaces[1,2,4]. The Siglec-sialic acid axis is a promising potential for immunotherapy and efforts are underway to develop therapies that block Siglec-sialic acid interactions[5-7]. However, there are major challenges associated with in determined precisely what are the specific carbohydrate ligands of Siglecs.

Carbohydrate ligands of Siglecs are difficult to study using conventional tools because (i) these interactions are biochemically weak and (ii) carbohydrate structures cannot be directly predicted from the genetic code. Advanced tools enhancing the avidity and stability of these interactions are needed to map Siglec-glycan landscapes on cancer cells.

These last years, the Macauley lab improved methods to detect Siglec ligands with the design of sensitive and quantitative methods and the use of recombinant soluble Siglecs[8-10]. Using these innovative tools in a flow cytometry-based application[11], we profiled Siglec ligands expression across cancer cell lines from the NCI-60 panel. We notably shown distinct Siglec binding patterns, providing insights into how cancer-specific sialylation signatures may influence Siglec interactions[8,9]. Accordingly, we got a 'fingerprint' of Siglec ligands on each type of cancer, which is currently used as a training dataset. Simultaneously, we are producing recombinant sialyltransferases to synthesize and study sialylated ligands in a controlled environment.

This currently facilitates the development of selective inhibitors of sialylation[12] and the biosynthesis of Siglec ligands on cell surface to validate sialyltransferases role in Siglec-sialic acid axis. We are combining the Siglec binding data generated with publicly available transcriptomics and proteomics data to predict the expression of what set of genes/proteins up regulate Siglec ligands. Overexpression and CRISPR-Cas9 knockout experiments[13] are currently developed to validate these predictions by assessing the impact of specific glyco-genes on Siglec ligand expression in cancer cells. From the predictive algorithms generated, we will conduct validation on clinical samples, particularly for patients not responding to current immunotherapies, which we hypothesize have higher levels of Siglec ligands.

This approach underscores the potential of targeting the Siglec-sialic acid axis to identify novel cancer biomarkers and improve personalized immunotherapy.

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(SPT8) Glycoengineering of CD8$^+$ T cell as a novel strategy to enhance T cell anti-tumour therapies

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T cell therapies have revolutionized cancer treatment. However, only a minority of solid cancer patients benefit from this therapeutic modality, highlighting the urgent need for identifying novel mechanisms underlying cancer immunoediting within the tumour microenvironment (TME). One of the reasons for non-response to immunotherapy in solid tumour is the complexity of TME that creates intricate immunosuppressive signals that result in the suppression of tumour-fighting T cells. Changes in glycosylation are a hallmark of cancer contributing to the suppressive TME. In line with this, the overexpression of tumour-associated branched *N*-glycans have been previously shown to play a pivotal role in cancer immunoediting by contributing to the creation of immunosuppressive networks in the TME[1]. In fact, T cells are extensively glycosylated and glycans are essential molecules in regulating T cell development, activity and function both in homeostasis and in inflammation[2,3].

However, the biological relevance of the T cell glycome as a regulatory layer of T cell activity and anti-tumour properties in TME remains largely unexplored. In this study, we demonstrated that intratumor T cell *N*-glycome composition is dynamically regulated in early stages of human colorectal cancer (CRC) development, displaying alterations in the expression of branched *N*-glycans in premalignant lesions of different CRC subtypes, imposing suppressive functions to T cells. Mechanistically, we demonstrated that CD8$^+$ T cells expressing branched *N*-glycans exhibit an exhausted phenotype with increased expression of PD1 and Tim3.

These results suggest an association of the expression of branched *N*-glycans in defining T cell properties in the TME associated with effector-memory *versus* exhausted programs. The deletion of this suppressive branched *N*-glycosylation layer on therapeutic CD8$^+$ T cells by CRISPR/Cas9, was able to prevent T cell exhaustion, imprinting an activated and anti-tumour phenotype. This was further demonstrated when these cells were co-cultured with cancer cells and injected in different CRC mouse models, in an antigen dependent manner, revealing increased cytotoxicity, through higher cellular degranulation, and increased tumour cell killing capability, contributing to the suppression of tumour growth and increased overall survival of the mice.

Together, these results unlock the power of T cell branched *N*-glycans as a new checkpoint that regulates T cell cytotoxicity in cancer, proposing the modulation of the T cell glycocalyx as an unprecedented opportunity to enhance cytotoxicity of T cells and the efficacy of immunotherapy in solid tumours.

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(SPT9) Neolactotetraosylceramide enables urinary detection of bladder cancer

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Tumour cells display a set of modifications that confers them selective advantages. Particularly, alterations in glycosylation pathways are a common feature of all cancer hallmark abilities, with most FDA-approved tumour markers being glycan-based. Bladder cancer (BC) is the 9th most common cancer worldwide[1].

Current diagnosis still relies on invasive methods, mainly cystoscopy. Regarding the non-invasive tests available, urine cytology is not reliable as a primary diagnostic tool, showing overall sensitivity and specificity of up to 48% and 86%, respectively[2]. Moreover, the existing tumour markers lack formal indication for clinical practice. Therefore, there is an urgent need to identify new biomarkers for the reliable detection of BC. Glycosphingolipids (GSLs) are potential biomarkers for cancer diagnostics.

Here, we applied glycan analytics by multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF)[3] to unravel the global GSL profile of tumour tissues and urine samples from bladder cancer patients. We detected neolactotetraosylceramide (nLc4, Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) at statistically significantly increased levels from tumorigenic regions compared to non-malignant adjacent tissue (n = 30), which was validated by immunofluorescence staining. xCGE-LIF analysis of urinary extracellular vesicles showed that nLc4 is increased in bladder cancer patients (n = 16) when compared to controls (n = 50; accuracy, 82%; AUC, 0.75). Finally, we set-up an ELISA targeting nLc4 in a discovery set and an independent validation set, comprising normal, low- and high-grade bladder cancer urine samples. Increased levels of nLc4 distinguished groups of patients with high grade bladder cancer from control subjects (discovery cohort: n = 18; accuracy, 85%; AUC, 0.94; validation cohort: n = 26; accuracy, 79%; AUC, 0.83).

In conclusion, nLc4 has potential as a urinary biomarker for the non-invasive detection of bladder cancer.

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(SPT10) Novel CAR-T formulations targeting tumour-associated glycoepitopes: A new strategy for solid tumours

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The accurate targeting of tumour-specific antigens is behind the success of any Chimeric Antigen Receptor (CAR) T cell therapy[1]. While cell surface antigens are rarely exclusively cancer-specific, their post-translational modifications (PTMs) offer a promising alternative. Indeed, the carbohydrate coat present on the surface of every living cell, which is altered in cancer, offers a variety of potential targets for immunotherapeutic approaches[2,3]. Among these, truncated *O*-glycans are potential candidates given their prevalent presence in various epithelial tumours and rare detection in healthy tissues[4,5].

In this study, we developed a novel short *O*-glycan-targeting monoclonal antibody (mAb) with unprecedent binding profile. This novel mAb specifically binds to epithelial tumours expressing the target antigen without reacting with healthy tissues. We identified the coding sequence of the novel mAb and engineered its corresponding single-chain variable fragment (scFv) into a second-generation CAR scaffold. The resulting CAR T cells effectively killed various solid cancer cell models in the *in vitro* setting. Additionally, these CAR T cells also eliminated patient-derived organoids (PDOs) from gastric cancer while sparing normal gastric mucosa-derived organoids. Notably, the novel CAR T formulation demonstrated robust control over various human cancer xenografts in mouse models, underscoring its efficacy in targeting and managing complex solid tumours.

Overall, our study introduces a novel, powerful, and precise glycan-directed CAR T cell therapy designed to target a broad spectrum of carcinomas, combining exceptional specificity and efficacy with a strong safety profile.

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(SPT11) Unraveling the role of terminal fucosylation in pneumococcal pneumonia

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Background: Mucosal tissue surfaces such as that of lungs are rich in *N*- and *O*-glycans containing terminal sugars such as fucose. Some individuals have mutations in the fucosyltransferase (FUT) genes (*FUT1*/*FUT2*), resulting in the inability to secrete α-(1,2)-fucosylated glycans. Such mutations render these individuals susceptible or resistant to infection by certain pathogens.

Aim: Investigation of how terminally fucosylated glycans influence *Streptococcus pneumoniae* (S.pn.) infection.

Methods: We employed a murine model of pneumococcal pneumonia using mice systemically treated with 2-deoxy-D-galactose (2d-Gal), a biochemical inhibitor of fucosylation. The murine glycome was evaluated using LC-MS/MS. Lung immune cell recruitment was analysed via flow cytometry. Pro-inflammatory cytokine concentrations and degree of lung permeability were determined through ELISA. Bacterial gene expression was analysed via RNA sequencing of S.pn. following co-culture with native MUC5AC or mucin glycans.

Findings: Fucosylated glycans are abundant in murine and human small airways. Fucose utilization genes are upregulated in S.pn. following incubation with natively purified gastric porcine MUC5AC *in vitro*. Fucosylated glycans are shed into the bronchoalveolar lavage (BAL) during S.pn. infection. Systemic treatment of mice with 2d-Gal resulted in incorporation of 2d-Gal into the murine glycome and inhibition of fucosylation *in vivo*. Mice treated with 2d-Gal displayed improved physiological parameters such as infection-induced body weight and temperature loss. Treatment with 2d-Gal led to reduced pulmonary bacterial burden in mice following S.pn. infection. Flow cytometry revealed that 2d-Gal treatment enabled quicker resolution of neutrophilic inflammation following infection. Lung permeability was reduced and lower IL-6 and TNF levels could be detected in BAL fluid of 2d-Gal-treated infected mice.

Conclusion: Restricting availability of fucosylated glycans may carry potential to limit S.pn. infectivity and subsequent pulmonary inflammation.

(SPT12) Dissecting Siglec-15 ligand expression in thyroid cancer

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Siglec-15 is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family and is expressed by human osteoclasts and macrophages. This receptor has emerged as a key regulator of tumour immunology and survival. Expression of Siglec-15 on tumour-associated macrophages (TAMs) suppresses anti-tumour immune responses, and stimulated proliferation of tumours[1]. Targeting Siglec-15 and its ligands holds potential for enhancing tumour immunity and immunotherapy[1]. Upon extracellular ligand binding, Siglec-15 recruits intracellular adapter proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs) through its positively charged transmembrane region[2]. This triggers downstream signalling cascades that influence osteoclast and macrophage function.

Various studies determined sialyl-Tn (Sia-α2,6-GalNAc-α1-O-Ser/Thr) glycans are a ligand for Siglec-15[3,4]. However, recent studies suggest that the glycan presentation on distinct glycoproteins, such as CD11b, provides additional binding context that directs Siglec-15 binding selectivity, even without sialyl-Tn glycans being present[3-5]. Nevertheless, the precise nature of a specific glycan context that results in biologically meaningful binding and downstream signalling of Siglec-15 remains unclear.

We have identified that thyroid cancer cells express glycan ligands for Siglec-15, and correlated high Siglec-15 expression with high TAMs infiltration and tumour aggressiveness. To dissect Siglec-15-ligand interactions in thyroid cancer, we used established human thyroid cancer cell lines and tested their endogenous expression of Siglec-15 ligands.

Next to that, we have developed human monocytic cell lines with stable Siglec-15 expression. Using glycosylation inhibitors and genetic engineering, we found that sialyl-Tn is not presented by the thyroid cancer cells, but that Siglec-15 ligands are formed by other O-glycan structures. Moreover, we have identified potential glycoprotein candidates that provide context for selective Siglec-15 binding. Recombinant production of these glycoprotein ligands using the cell-based glycan array now allows us to perform binding studies with Siglec-15 expressing monocytes and macrophages, and to analyse downstream signalling pathways and immune effects.

We will share our insights into Siglec-15-ligand interactions in thyroid cancer, which may aid the development of novel strategies to target this emerging immune checkpoint.

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(SPT13) Mucin degrading enzymes – a platform to study their activity

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Mucus is one of the first physical barriers to systemic delivery for oral and pulmonary therapeutics[1]. The physiological role of mucus and epithelial mucins is largely to provide a barrier to separate the harsh outer environment from the epithelial cell surface. It has evolved to trap potentially pathogenic organisms such as bacteria and viruses by steric effects in addition to electrostatic and hydrophobic interactions. The high clearance rate of mucus, up to 10L per day in the small intestine, means that trapped species are quickly excreted emphasising the need for fast transport through this barrier.

The significance of this for new modalities such as peptides, oligonucleotide and protein therapeutics is increasingly being recognised. Current mucolytic approaches include reducing agents and DNases, which have both been investigated with limited success[2]. None of these target the long protein backbone filaments of mucin which make up the mesh-like structure of mucin. These long strands, which even in the reduced form can extend several hundreds of nanometres, are typically resistant to most proteolytic degradation due to their characteristic heavy O-glycosylation.

A specific class of enzyme, mucinases, can degrade this mucin backbone to further break the long mucin filaments down into small fragments[3]. We have recently shown the promise of these mucinases, for the first time, in oral drug delivery. However, engineering these mucinases holds further potential for increased mucin subtype specificity. In this work a platform based on cell surface display is presented to investigate their activity in a defined environment and as a platform for mucinase engineering[4]. This can reveal both mucinase activity in different physiological fluids and is being applied to engineer these enzymes for improved biological activity and specificity. As shown in Figure 1A, the platform is based on the cell-surface display of recombinant mucins. The incorporation of a fluorescent marker at the distal end allows for identification of cleavage by flow cytometry or fluorescence well-plate reader (Figure 1B).

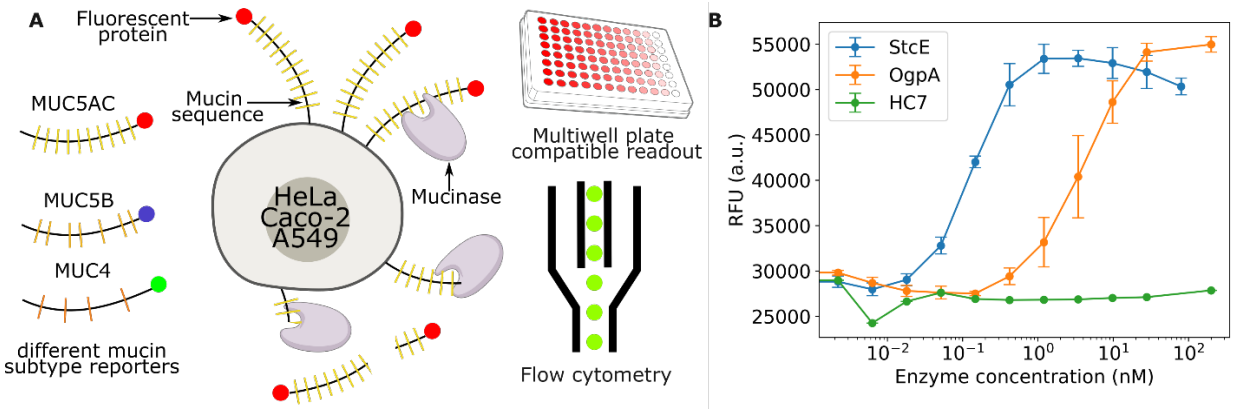

Figure 1: A) Schematic overview of the cell surface display platform and B) illustrative readout of enzymatic activity using the developed platform with 3 different mucinases with different activity.

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(SPT14) Development and Testing of Novel Small Molecule Inhibitors for the Manipulation of IgE Glycosylation

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Immunoglobulin E (IgE)-based antibodies are currently under investigation as promising novel cancer therapeutics. Structure-function studies are complicated by the structural complexity of the IgE glycoforms. Using small molecule inhibitors, with established cell cultures, for manipulating glycosylation is a complementary approach to the genetic disruption of glycan biosynthesis. This would provide a novel method for IgE glycoengineering, establishing a basis for structure-function relationship studies of IgE and optimizing its biological and therapeutic properties.

Here, we report results from IgE glycoengineering with three commercial glycosidase inhibitors and two novel glycosyltransferase inhibitors which were added, separately, into IgE-secreting cell cultures. The initial data indicates commercial inhibitors targeting late stage glycan biosynthesis steps are poorly tolerated and have limited impact on IgE glycan composition whereas commercial inhibitors targeting early-stage glycan biosynthesis steps tend to be better tolerated and have broader effects on IgE glycosylation. Comparatively, tolerance within expression systems of our novel glycosyltransferase inhibitors varied as did overall impact on glycosylation. Whilst glycan complexity was decreased to a limited extent, overall changes to the IgE glycome profile were minimal.

Although the glycosyltransferase inhibitors had a limited effect on modifying IgE glycans, our data partially support the effectiveness of our concept. To further validate these findings, additional functional experiments are ongoing.

(SPT15) ST6GAL1 role in antibody therapy response and immune evasion of colorectal cancer

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Targeted therapies of colorectal cancer, including immune checkpoint inhibitors, have been applied to boost native anti-tumour responses and improve patient survival. However, most patients still face a dismal prognosis, highlighting the need to identify novel immunotherapeutic targets to optimize treatment outcomes. Glycosylation is a highly controlled cellular process that is deregulated in cancer cells, with aberrant expression of specific glycans such as terminal sialylated structures. Aberrant glycan signatures have also been reported in cancer-derived extracellular vesicles (EVs), that are released by the cells into the extracellular space, and constitute important mediators of intercellular communication. The terminal sialylated aberrant glycans are known to be recognized by immune cells through sialic acid binding-immunoglobulin-like lectins, Siglecs, inducing strong inhibitory signalling and therefore suppressing immune cell functions [1,2]. This study aims to investigate the role of ST6Gal1 in modulating antibody therapy response and crosstalk between colorectal cancer and immune cells.

We have disclosed the importance of terminal sialylation, displayed in EGFR tyrosine kinase receptor, as therapeutic target in colorectal cancer, showing that ST6Gal1 can underpin the clinical performance of approved targeted therapeutic antibodies and patient response to therapy, impacting cancer patients' clinical outcome [3]. Taking advantage of the developed genetic engineered cancer cell models with differential expression of the glycosyltransferase ST6Gal1 and terminal sialylated glycans profile, we identified Siglec-10 receptor as a binding partner for glycans synthesized by ST6GAL1 in cancer cells. *SIGLEC10* was found to be expressed in subsets of tumour-associated myeloid cells, mainly macrophages. Analysis of the functional consequences of sialylated glycans on immune response have shown that terminal sialylation shifts macrophage:tumour cells ratios in co-culture and regulates macrophage-mediated phagocytosis of cancer cells. Furthermore, we showed, through abrogation by CRISPR/Cas9 of Siglec-10 on immune cells, that this mechanism was dependent on a Siglec-10 circuit activation. These findings disclosed ST6Gal1/Siglec-10 axis as a key glyco-immune checkpoint involved in tumour immune evasion in colorectal tumours, with potential as a novel cancer immunotherapeutic strategy. Moreover, we observed that ST6Gal1 is enriched in cancer-derived EVs from colorectal cancer cells and demonstrated that cancer-derived EVs glycosylation signatures modulate the immune response and promote a pro-tumorigenic environment.

These results uncover ST6Gal1 as a regulator in therapy response and tumour immune evasion in colorectal cancer, providing novel immunotherapeutic clinical targets and improving cancer patient stratification to optimize treatment outcomes.

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(SPT16) Endometriosis specific vaginal microbiota links to urine and serum *N*-glycome

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Endometriosis is a chronic systemic disease in which tissue similar to uterine lining grows outside the uterus. This condition affects approximately 10% of reproductive-aged women worldwide. Its aetiology is poorly understood, and no long-term cure is available. The development and persistence of the disease depend on several coexisting factors. The vaginal microbiome is one of these factors, but its role in endometriosis and its systemic involvement is not fully understood.

Here we investigated the vaginal microbiota, serum and urine glycome and immunoglobulin G glycosylation in endometriosis patients in comparison with controls - infertility patients with similar complaints but no clinical evidence of endometriosis. We identified an endometriosis-specific vaginal microbiota, that was not present in controls. Endometriosis patients exhibited a loss of the dominant *Lactobacillus* species, *Lactobacillus iners*, and presented increased bacterial diversity, including species such as *Anaerococcus senegalensis, Anaerococcus octavius, Prevotella jejuni* and *Porphyromonas bennonis*. The levels of trigalactosylated and triantennary serum glycans, as well as core fucosylated mono-antennary glycans from urine IgG correlated with the levels of *A. senegalensis* in endometriosis patients. Although urine glycans did not significantly differ in endometriosis, they contained four novel sulfated glycans distinct from serum IgG, indicating functional relevance.

Our findings contribute to a deeper understanding of the relationships between the vaginal microbiota and the serum and urine glycome in endometriosis. The link between the local vaginal microbial environment and the systemic nature of endometriosis is evident in the altered urine and serum glycans which correlate with vaginal microbial species. Further investigation of microbiota identification and diversity typing in conjunction with host and clinical factors, will refine the diagnosis and understanding of the aetiology of this enigmatic chronic disease. Further functional studies based on these findings are warranted.

(SPT17) Validation of the glycomics-based GlycoCirrhoTest as predictor of risk of HCC development in cirrhosis

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Background: Cirrhosis is the main risk factor for Hepatocellular carcinoma (HCC). Six-monthly ultrasound screening is the current practice for surveillance, but policies are shifting towards a more personalized approach. We previously demonstrated that GlycoCirrhoTest, a biomarker based on the serum N-glycome of patients, provides information regarding the risk of HCC development, with a near-zero 5-year risk for HCC for those cirrhosis patients that test negative on GlycoCirrhoTest[1], and a very strong risk enhancement for those who test positive. The present new study independently validates GlycoCirrhoTest for HCC risk-assessment in cirrhosis, in a cohort that reflects the current disease aetiology composition of routine hepatology practice, in contrast to our earlier studies which were dominated by patients with chronic HCV infection aetiology, which is now gradually disappearing in the European advanced liver disease patient population thanks to the widespread use of highly effective direct-acting antiviral agent therapy.

Methods: This study is a prognostic biomarker validation on 197 patients with compensated cirrhosis (CHILD-Pugh A/B) in a tertiary liver centre. GlycoCirrhoTest (GCT)[1,2] measures the ratio of biantennary glycans with bisecting GlcNAc over triantennary glycans in the total sialidase-treated serum proteome-linked *N*-glycome. The *N*-glycome is profiled by CGE-LIF (capillary gel electrophoresis with laser-induced fluorescence) on the clinical electrophoresis diagnostic platform that is also used for many DNA separation-based molecular pathology tests. We recorded the patient's baseline GlycoCirrhoTest and during the follow up, patients underwent 6-monthly screening for HCC with ultrasound.

Results: 19 patients developed HCC during 7 years of follow up. At baseline, the mean GlycoCirrhoTest value was significantly higher in patients who developed HCC within 3 years compared to patients who did not. A high baseline GlycoCirrhoTest was associated with increased HCC incidence: Hazard Ratio (HR) of 5.9 (95% CI: 0.7 – 48), 4.5 (95% CI: 1.3 – 15.6) and 3.4 (95% CI: 1.1 – 10.3) at 3, 5 and 7 years post sampling. The test is clinically most useful to rule out high risk of HCC. These results are in agreement with our previous results[1], illustrated in a meta-analysis. We could also confirm that albumin and age are independent predictors for HCC. Albumin blood levels < 4 g/dL are associated with an increased HCC incidence, illustrated by a HR at 7 years of 2.4 (95% CI: 1.1 – 5.2). For subjects with both high GlycoCirrhoTest and low albumin we found a HR of 11.1 (95% CI: 3.9 to 31.7) at 7 years.

Conclusions: GlycoCirrhoTest provides additional information for risk assessment of HCC development in cirrhosis. This could be used to devise personalised HCC screening programs in cirrhotic patients. Serum albumin levels and age provide additional, GCT-independent information which may add to the utility of the test.

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(SPT18) Chemoenzymatic strategy for single glycoforms monoclonal antibody production

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Monoclonal antibodies (mAbs) are the most widely used protein therapeutics in the treatment of cancers, arthritis, and other human disorders worldwide. Currently the production of mAbs by mammalian (e.g. CHO cells) or alternative cell culture platforms gives rise to heterogenous mAb structures[1]. Contrastingly, homogenous mAbs demonstrate higher specificity and selectivity to their ligands (FcRs)[2]. The value of this is that the patient dose can be decreased for the same clinical outcome and consequently the value of the antibody can be enhanced. *N*-glycans have the ability to fine tune immunological responses such as antibody-dependent cellular cytotoxicity (ADCC) or anti-inflammatory properties. The formation of these highly useful single glycoforms still remains a challenge.

Two current glycoengineering methods include a) cell line engineering and b) media supplementation and process parameter alterations[1]. Here we present an alternative chemoenzymatic workflow for the design and characterisation of a single glycoforms mAbs using a previously developed *N*-glycoanalytical technology[3,4]. In brief, the workflow sequentially removes key glycan motifs such as sialic acids, galactose, *N*-acetylglucosamine and fucose residues from the mAb originator molecules followed by addition of selective glycan epitopes utilizing glycosyltransferase enzymes and sugar donors to generate a family of glycoengineered mAbs for improved pharmacokinetics and enhanced effector functions.

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(SPT19) *De novo* sequencing of human milk oligosaccharides using IM-MS

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Human milk is the gold standard for an infant's nutrition. It consists of bioactive substances such as lipids, proteins, and human milk oligosaccharides (HMOs). These HMOs are crucial for protection against infections and for the development of the intestinal microbiome. To establish structure-function relationships and exploit glycoscience for the development of future nutraceuticals and therapeutics, it is essential to determine exact HMO structures in human milk. Currently, accurate mass measurements are used for compositional assignment and additional MS/MS fragmentation experiments can provide structural information. Due to their isomeric nature, however, the assignment of exact HMO structures from MS/MS spectra remains very challenging. Furthermore, the lack of well-defined standards is another major hurdle for exact structure identification. In this work, we present an ion mobility-MS de novo sequencing methodology, that can unambiguously determine isomeric HMO structures in the absence of synthetic standards.

A limited set of chemoenzymatically synthesized isomeric HMOs was used to develop a drift tube ion mobility spectrometry (DTIMS)-MS methodology and a reference database with common terminal glycan epitopes, including Lewis and human blood group epitopes, for *de novo* sequencing of isomeric HMO structures. The DTIMS-MS methodology uses high-resolution demultiplexing to obtain high-resolution arrival time distributions (ATDs; up to 240 $\Omega/\Delta\Omega$) and accurate mass and CCS values of intact isomeric HMOs and their fragments.

The DTIMS-MS methodology was used for the elucidation of HMO structures with undefined carbohydrate sequences and linkages in neutral and acidic fractions of milk donors. Fragments of HMOs were identified by their accurate mass and CCS values, using fragment ion entries from the reference database, and used for *de novo* sequence assembly to elucidate the HMO structures. The CCS values of elucidated intact structures were then added to the reference database and used to identify larger and more complex isomeric structures, creating a self-expanding reference database in the absence of further standards. This resulted in the elucidation of exact HMO structures in the samples, ranging up to a degree of polymerization of 9, and >250 new (fragment) ion entries for the reference database. The structure assignment by DTIMS-MS was validated with well-defined standards and revealed 100% correct assignment by the *de novo* sequencing method.

This new methodology can also be applied to other glycan classes. It will allow the glyco-workfield to rapidly and unambiguously identify exact structures in biological samples, independently of reducing end labels, without the need for synthetic standards.

Posters

(P01) Glycoengineering of CD8$^+$ T cell as a novel strategy to enhance t cell anti-tumour therapies

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T cell therapies have revolutionized cancer treatment. However, only a minority of solid cancer patients benefit from this therapeutic modality, highlighting the urgent need for identifying novel mechanisms underlying cancer immunoediting within the tumour microenvironment (TME). One of the reasons for non-response to immunotherapy in solid tumour is the complexity of TME that creates intricate immunosuppressive signals that result in the suppression of tumour-fighting T cells. Changes in glycosylation are a hallmark of cancer contributing to the suppressive TME. In line with this, the overexpression of tumour-associated branched *N*-glycans have been previously shown to play a pivotal role in cancer immunoediting by contributing to the creation of immunosuppressive networks in the TME[1]. In fact, T cells are extensively glycosylated and glycans are essential molecules in regulating T cell development, activity and function both in homeostasis and in inflammation[2,3].

However, the biological relevance of the T cell glycome as a regulatory layer of T cell activity and anti-tumour properties in TME remains largely unexplored. In this study, we demonstrated that intratumor T cell *N*-glycome composition is dynamically regulated in early stages of human colorectal cancer (CRC) development, displaying alterations in the expression of branched *N*-glycans in premalignant lesions of different CRC subtypes, imposing suppressive functions to T cells. Mechanistically, we demonstrated that CD8$^+$ T cells expressing branched *N*-glycans exhibit an exhausted phenotype with increased expression of PD1 and Tim3.

These results suggest an association of the expression of branched *N*-glycans in defining T cell properties in the TME associated with effector-memory *versus* exhausted programs. The deletion of this suppressive branched *N*-glycosylation layer on therapeutic CD8$^+$ T cells by CRISPR/Cas9, was able to prevent T cell exhaustion, imprinting an activated and anti-tumour phenotype. This was further demonstrated when these cells were co-cultured with cancer cells and injected in different CRC mouse models, in an antigen dependent manner, revealing increased cytotoxicity, through higher cellular degranulation, and increased tumour cell killing capability, contributing to the suppression of tumour growth and increased overall survival of the mice.

Together, these results unlock the power of T cell branched *N*-glycans as a new checkpoint that regulates T cell cytotoxicity in cancer, proposing the modulation of the T cell glycocalyx as an unprecedented opportunity to enhance cytotoxicity of T cells and the efficacy of immunotherapy in solid tumours.

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(P02) *N*-glycosylation profiling of natural killer cells upon activation

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With the evolution of adoptive cell therapies for advanced malignancies, such as Chimeric Antigen Receptor T cell (CAR-T) therapy and the emerging development of natural killer (NK) cells expressing CARs, there is a growing need to understand the impact of glycosylation on immune cell function.

Glycosylation, the addition of chains of monosaccharides, to proteins, lipids and other biomolecules, is a ubiquitous process controlled by a balanced expression of glycosyltransferases and glycosidases. *N*-glycans are a class of glycan that attach to newly translated proteins at asparagine residues (Asn-X-Ser/Thr) during early protein folding in the endoplasmic reticulum (ER) and subsequent processing within the Golgi apparatus.

N-glycans on T-cell receptors function to provide locus stabilisation on lipid rafts prevent sub-threshold activation[1]. Several *N*-glycans types have been identified in cell-to-cell signalling, potentially including immunological interactions. *N*-Glycans with terminal polylactosamine chains serve as binding sites for carbohydrate binding proteins in the Galectin family[2]. These binding interactions regulate a wide range of biological processes including inflammation and influence the immune response to, and growth of several cancers.

A recent bioinformatic study in this laboratory, confirmed upregulation of glycosyltransferase transcripts in NK cells after cytokine stimulation. In addition, there were transcriptional changes hinting at increased formation of polylactosamine (polyLac) chains that may participate in NK cell-to-cell signalling during immune activation. This study we will present describes a robust method of measuring glycan profile changes in intact NK cells under different stimulation conditions; comparing the retrieval between whole intact cells, where glycans are harvested from the cell membrane surface, and whole cell proteome after lysis buffer-based protein extraction.

Our workflow utilised a feeder cell-based protocol to expand human NK cells from peripheral blood mononuclear cells (PBMCs) and we validated their functional activities following 14 days of expansion. Upon confirmation of successful expansion (achieving >85% purity of NK cells at day 14) and functional capacity, the cells were submitted to glycan analysis by 2 optimisation methods. *N*-glycan retrieval was performed by preparation and enzymatic cleavage using Peptide-*N*-glycosidase F (PNGase F) directly from intact NK cells, and this was compared to the *N*-glycans obtained from whole cell protein extraction using a previously established workflow[3]. Glycan retrieval was performed upon NK cells before the expansion process by NK cell isolation from PBMCs and compared to Day 14 expanded cells. In addition, upon confirmation of functional competence, the NK cells were analysed for changes in the glycan profile when subjected to cytokine stimulation with IL-12 and IL-15, untransfected K562s and a combination of both.

Our results provide novel methods to interrogate cell surface-specific *N*-glycosylation changes on human immune cells and demonstrate the dynamic nature of the glycome of activated lymphocyte subpopulations.

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(P03) Decoding molecular changes in glucosylceramide synthase-deficient pluripotent stem cells via multi-omics profiling

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Glycosphingolipids (GSLs) are ubiquitously expressed, complex glycosylated membrane lipids. They are involved in basic cellular processes and can be linked to various diseases [1,2]. While GSLs are not essential in embryonic stem cells [3], the diversity of GSLs is important during embryogenesis, where different GSLs are expressed at specific stages and along several differentiation pathways.

With the aim to study their relevance for early mammalian development, we mutated the UDP-glucose ceramide glucosyltransferase gene (*UGCG*) encoding the enzyme glucosylceramide synthase (UGCG) in human induced pluripotent stem cells (hiPSCs) by CRSIPR/Cas9. The functional impairment of UGCG was confirmed by glycomic profiling using multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence detection (xCGE-LIF)[4]. Notably, UGCG-KO hiPSCs displayed normal morphology, growth behaviour, and expression of stem cell markers compared to wildtype (WT) hiPSCs. Differentiation into early progenitors of meso-, endo-, and ectoderm along with analysis of differentiation efficacy using characteristic cell surface markers, revealed similar proportions of positive cell populations in UGCG-KO and WT hiPSCs.

To explore potential differences of UGCG-KO hiPSCs in a multi-cell-type context and to investigate their pluripotency *in vivo*, we conducted teratoma assays. Pluripotency of the UGCG-KO hiPSC line was confirmed; however, no discernible differences were observed compared to WT hiPSCs. Comparative quantitative proteomic profiling of WT and UGCG-KO explanted teratomas revealed significant differences for 295 proteins, with significant enrichment of proteins related to protein localization, protein transport, signal transduction, and organelle localization processes.

Complementary transcriptomic profiling further showed that transcript levels of numerous genes were significantly affected by the UGCG-KO in hiPSCs and derived ectodermal derivatives. Especially C-type lectin domain family 2 member A (CLEC2A) transcription was significantly lower in UGCG-KO compared to WT derived cells. Given that UGCG impairment likely disrupts lipid homeostasis, we conducted global lipidomic profiling by mass spectrometry to evaluate the impact on 24 distinct membrane lipid classes. This analysis revealed a significant increase in sphingomyelin levels in UGCG-KO hiPSCs.

In summary, our study clearly demonstrates that the lack of GSLs affects hiPSCs at the glycomic, transcriptomic, proteomic, and lipidomic levels. Although these effects do not seem relevant for pluripotency, it is hardly to imagine that GSLs are unnecessary at the pluripotent stem cell level. Further investigation is needed to elucidate their specific functions.

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(P04) Lubricin glycosylation in tears derived from patients with dry eye disease

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Dry eye disease (DED) is a multifactorial condition characterized by tear film instability, inflammation, and ocular surface damage[1,2]. It is classified into aqueous-deficient and evaporative types, both leading to tear film dysfunction[3].

Tears have gained significant relevance as a source of biomarkers for ocular and systemic diseases and can be collected through non-invasive methods[4,5]. Tears are a complex fluid that can be considered an intermediate between plasma serum and cerebrospinal fluid. They contain diverse components, including proteins, lipids, small metabolites, miRNAs and exosomes[5-8]. There are three types of tears: basal, reflex, and emotional. Basal tears, which are autonomously secreted, have the highest concentration of biomolecules[9]. These are contained in the tear film, a three-layered structure consisting of a superficial lipid layer, a middle aqueous layer, and an inner mucin layer[10].

While the mucus and aqueous layers were traditionally considered separate, recent studies suggest they form a single layer called glycocalyx[11], which contains a coat of glycans, trans-membrane glycoproteins, and glycoconjugates. The glycocalyx is the boundary between the aqueous layer and the mucin layer and plays a crucial role in the integrity and function of the ocular surface[12].

Lubricin, also known as proteoglycan 4 (PRG4), is a key component of the glycocalyx. As a mucin-like proteoglycan with extensive O-linked glycosylation, it plays a crucial role in maintaining ocular surface integrity[13]. Recent studies on the glycosylation profile of lubricin in synovial joint health and disease suggest that its glycosylation patterns can serve as early-stage biomarkers[14].

This study aimed to identify differences in lubricin glycosylation in tears between DED patients and healthy controls for early diagnosis. Tears were collected using Schirmer strips, and lubricin glycosylation patterns were assessed in a small set of clinical samples using western blot and a validated lectin assay[14].

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(P05) Synthesis and characterisation of a xeno-glycan based immunomodulatory hydrogel

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Introduction: An appropriate inflammatory response at the site of injury plays a vital role in tissue remodelling and regeneration. During the early stages of injury, pro-inflammatory cytokines are elevated, facilitating debris clearance through phagocytosis. In the later stage, anti-inflammatory cytokines predominate, promoting tissue regeneration and leading to fibrotic scarring[1]. Recently, biomaterials incorporating immunomodulatory agents have emerged as a promising approach to influencing tissue regeneration[2,3]. However, these strategies often have short-lived effects due to rapid degradation or transient immune activation. To address this limitation, we propose leveraging the immune system's natural recognition of foreign elements using xeno-glycans to modulate immune responses in a controlled manner. We hypothesize that an in situ forming α-Gal functionalized elastin-like recombinamer-based hydrogel, fabricated via catalyst-free click reactions, can promote endogenous tissue remodelling and functional recovery through immunomodulation[4].

Methods: Elastin-like recombinamers (ELRs) are produced using recombinant DNA technology and further modified specifically with azide and strained alkyne groups. Alpha-Gal was conjugated to ELRs with click cycloaddition in different stoichiometry. After conjugation, the binding affinity of Alpha-Gal towards human serum IgG, IgM, and m86 anti-alpha-Gal was evaluated by indirect ELISA and microscale thermophoresis (MST). Furthermore, Alkyne and azide groups of ELRs were utilized to form hydrogel. Human macrophages were treated with the hydrogel to study the polarization state by evaluation of secreted cytokines and gene analysis at different timepoints via ELISA and RT-PCR.

Results: The α-Gal epitope on the ELR was specifically recognized by the m86 anti-α-Gal antibody and human serum IgG and IgM. MST showed a higher binding affinity (low KD) towards α-Gal ELRs. Rheological assessments of the gel revealed its viscoelastic nature, and the nanomechanical indentation measurements on the ELR gel gave Young's modulus values of 1500 – 2000 Pa, indicating a soft hydrogel. Macrophages seeded on top of the ELRs showed a sustained release of TNF-α and IL – 10. RT-PCR showed upregulation of proinflammatory genes, such as CXCL10, CXCL8, and VEGFA and anti-inflammatory genes like IL10, IL-1Ra.

Conclusions: By conjugating the α-Gal epitope to ELR, we fabricated a xeno-glycan-functionalized ECM-based soft hydrogel that specifically binds to antibodies, which could lead to complement activation. The gel further modulated the polarization state of macrophages.

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(P06) Glycomic characterization of stratified ischemic human stroke samples obtained from brain banks.

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Stroke is a leading cause of mortality and disability worldwide. Currently available treatments aim to minimize neuronal damage in the acute phase but do not comprehensively address the molecular processes involved in post-stroke tissue remodelling[1]. Despite advances in neuroscience, the mechanisms regulating this remodelling are still not fully understood.

Glycosylation, a key post-translational modification, plays a fundamental role in cell communication, inflammation, and neuronal repair. Previous studies on brain glycosylation[2] have demonstrated significant changes in glycan patterns in neurodegenerative diseases like Parkinson's disease, providing insights into how glycosylation contributes to disease progression and tissue remodelling. In this study, we performed a detailed glycomic analysis of human brain tissue samples with ischemic stroke, obtained from brain banks (REC Application Reference Number: 2023.10.015), focusing on the *N*-glycosylation profiles at distinct phases of stroke progression: acute (<24 hours), subacute (25 hours to 5 days), and chronic (>6 days). The samples were collected from ***n=6*** sex-matched individuals aged 30-100 years, including both sexes, diagnosed with ischemic stroke. The affected brain tissue was primarily sourced from regions irrigated by the anterior circulation, with a preference for parietal and temporal lobes. To this end, we used ultra-performance liquid chromatography (UPLC)-mass spectrometry to spatially characterize *N*-glycans, lectin histochemistry to visualize specific glycan motifs, and immunohistochemistry to detect key proteins involved in post-infarction remodelling.

The results of this study revealed distinctive glycomic signatures associated with the progression of cerebral infarction, offering new insights into the role of glycosylation in this pathophysiology. These findings could contribute to the development of glycan biomarkers and potential therapeutic targets, highlighting the importance of translational glycoscience for understanding cerebrovascular diseases and their application in precision medicine.

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(P07) Decoding glycan structures: The impact of diet and lifestyle on IgG and C3 *N*-glycosylation

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Immunoglobulin G (IgG) and the complement component C3 are serum glycoproteins and central components of the immune system, whose glycosylation influence their immune functions and is altered in inflammatory, autoimmune and metabolic diseases[1,2]. Previous studies have shown that glycosylation patterns of both IgG and C3 indicate an organism's inflammatory state. Additionally, different dietary components and lifestyle factors may have pro- or anti-inflammatory effects on the body.

Therefore, in a general population of 800 adults from Croatia, we investigated the N-glycosylation of IgG and C3 in the context of diet and lifestyle. Glycan composition, including the specific sugar structures on IgG and C3, was determined using high-throughput LC-MS approaches, which involved enzymatic digestion followed by the analysis of the resulting glycopeptides. The relative glycan composition at each glycosylation site of a specific protein was quantified. Glycan data were examined, and after pre-processing, non-negative matrix factorization (NMF) was applied as a dimensionality reduction technique.

Following this, correlation analysis using Kendall's method was performed to assess the relationships between lifestyle factors and the compressed glycosylation profiles. Correlation analyses revealed that two NMF components were associated with existing data on the glycosylation of IgG and the C3, depending on dietary and lifestyle factors. Furthermore, each of these two components represented specific types of glycans, as determined by their respective weight factors. For IgG, the component that primarily represented less galactosylated glycans, with core fucose, and lacking sialic acids (G0F, G0FN, and G1F), exhibited a positive correlation (ranging from 0.27 to 0.21) with the consumption of salty snacks, serum HbA1c, number of deceased siblings, serum glucose, number of marriages and going to work by car. It also showed a weak positive correlation with consumption of chocolate, serum uric acid, number of children, BMI and consumption of muesli and salami (0.20 to 0.18). Glycans represented by this component are known to be associated with various inflammatory and autoimmune diseases, as well as different inflammatory markers such as CRP and IL-6[1]. For the C3 protein, one NMF component, which mainly represented high-mannose glycans with more mannose units as well as a glucosylated form (N2H9 and N2H10), from the Asn939 binding site, showed a positive correlation with body weight (0.31). It was also associated with serum metabolic parameters, including uric acid, VLDL, triglycerides, and glucose, as well as white wine consumption, with correlations ranging from 0.30 to 0.18. These glycan structures on C3 are known to be associated with type 1 diabetes, as children with T1D exhibit a higher proportion of unprocessed glycans[2].

The correlation between certain high-risk dietary and lifestyle factors or elevated serum parameters with proinflammatory or diabetogenic glycans supports their potential as markers of environmental influences on an organism. This insight could enhance individualised healthcare approaches and support the investigation of metabolic pathways involved in the development of different diseases.

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(P08) Fractalkine Mucin Domain Glycosylation Controls Trans-endothelial Migration of Monocytes

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Fractalkine (FKN) is a protein expressed on the surface of endothelial cells with a densely O-glycosylated mucin stalk capped by a chemokine domain. The chemokine domain is known to bind CX_3CR1, a receptor expressed by monocytes.[1] FKN-CX_3CR1 binding is necessary for monocytes to adhere to and penetrate the endothelium, a process known as trans-endothelial migration (TEM). Previous work has argued that FKN's chemokine domain is responsible for driving TEM and that the mucin domain is merely a structural feature that allows the chemokine domain to reach circulating monocytes;[2] however, the effect of mucin O-glycosylation on FKN function is underexplored.

Given this gap in knowledge, we sought to explore the role of the O-glycosylation in FKN function. To this goal, we found that when FKN glycosylation was ablated while retaining the mucin domain, we observed similar monocyte adhesion to endothelial cells as wild type FKN, yet significantly reduced TEM. This result suggested that FKN's chemokine domain is sufficient for monocyte-endothelium adhesion, in agreement with canonical understanding, but glycosylation of the mucin domain likely plays a role in remodelling the endothelium to allow for monocyte penetration beyond what is currently known.

To better understand this result, we performed the first glycoproteomic sequencing of FKN. We identified eleven unique N-glycan structures in the chemokine domain and hundreds of localized O-glycans occupying 49 glycosites in the mucin domain. We hypothesized that electrostatic attraction between the sialylated glycans in the mucin domain and the positively charged amino acids in the chemokine domain enable FKN to aggregate when bound to CX_3CR1, which activates a signalling cascade in the endothelium that allows monocytes to penetrate. This hypothesis was supported by fluorescence microscopy, which showed CX_3CR1- and FKN mucin glycosylation-dependent aggregate formation. Our findings showcase the potential of mucin O-glycosylation in signal transduction and further our understanding of immune health.

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(P09) Low abundant, difficult-to-analyse *N*-glycans of blood-derived human IgG - Where they are and how to find them

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Immunoglobulin G (IgG) is the highest abundant immunoglobulin in human blood. It plays a central role in the immune system by recognizing antigens and mediating effector functions of the humoral immune defense. These include complement dependent cytotoxicity, antibody dependent cell-mediated cytotoxicity, phagocytosis, and anti-inflammatory responses. The role of IgG *N*-glycosylation in many of these processes is well understood, in particular that of the conserved *N*-glycosylation site within the Fc part. In contrast, the Fab glycosylation is less well understood. Low abundant *N*-glycans with special features like sulfation or bisecting *N*-acetyllactosamine (LacNAc) were already identified in separate studies of blood-derived human IgG[1,2]. These structures are challenging to identify, usually require dedicated enrichment or analysis strategies, and they were not assigned to either the Fc or Fab yet. For these reasons, their occurrence on IgG is either not widely known or even actively disputed within the scientific community. Thus, these *N*-glycans are strongly underrepresented in studies of IgG *N*-glycosylation since their first discoveries.

Here, motivated by our previous work on the analysis of sulfated *N*-glycans[3–6], we present additional evidence for *N*-glycans bearing sulfation or bisecting LacNAc on IgG in a single analysis. Using simple and readily available methods, we localized these structures by combining proteolytic fragmentation of IgG into Fab and Fc, and exoglycosidase sequencing based on multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF). Further, we ultimately prove sulfation of Fab-derived *N*-glycans using a sulfatase that we have previously isolated and characterized for that purpose[4]. We used this enzyme in an epitope-directed glycan enrichment (EDGE-) profiling workflow. Herein, the sulfatase in its apo-state, lacking its cofactor for substrate desulfation, retains the capacity to bind sulfated *N*-acetylglucosamine. Thereby, sulfated *N*-glycans are enriched and separated from non-sulfated *N*-glycans, proving their modification with a sulphate group. Doing so, we established an intriguingly selective localization of *N*-glycans with sulfation or bisecting LacNAc on the Fab. Therefore, we anticipate and advocate that these structural features will receive more attention in future functional studies of IgG *N*-glycosylation and in biomarker discovery.

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(P10) 4F-GALNAC as a novel inhibitor of cancer-associated *O*-glycans in gastrointestinal cancer cells

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The aberrant expression of sialylated *O*-glycans, such as *O*-linked SLeX and STn antigens, is a common feature in gastrointestinal cancer (GI). Thus, targeting these cancer-associated *O*-glycans biosynthesis has great potential for developing new therapies[1]. Recent studies have shown the efficacy of various fluorinated monosaccharide analogues as glycan biosynthesis inhibitors[2].

Hence, this work aims to evaluate the capacity of different fluorinated N-acetylgalactosamine (GalNAc) analogues, in impairing the biosynthesis of these aberrant *O*-glycans in GI cancer cells. We tested three different fluorinated GalNAc monosaccharides, with a fluorine atom at the positions -3, -4, or -6 (3F-, 4F- and 6F-GalNAc), in natural-expressing SLeX and STn glycoengineered (ΔC1GalT1) cell models. Furthermore, we tested their biosynthesis capacity and their impact on the biological characteristics of cancer cells. Overall, our results demonstrated that 4F-GalNAc reduced the immunodetection of both *O*-linked SLeX and STn antigens.

In addition, RNAseq analysis showed alterations in *N*-glycan biosynthesis and sugar donor metabolic activity. 4F-GalNAc was also shown to reduce the motility and invasive capacity of cancer cells, impact cell metabolism and E-selectin binding in both SLeX and STn-expressing cancer cells. *In vivo* results showed the capacity of 4F-GalNAc to reduce tumour growth in a xenograft mouse model.

Altogether, our results show that 4F-GalNAc can impair cancer-associated O-glycans biosynthesis and affect the cells' biological behaviour, constituting a promising novel approach for developing a new therapy.

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(P11) The Siglec-6 binding mechanism hinges on interactions with the cell membrane

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Sialic acid-binding immunoglobulin-type lectins (Siglecs) play a key role in regulating immune responses by recognizing specific sugar epitopes containing sialic acid (Neu5Ac). Siglecs are divided into two groups based on genetic similarity. Siglecs in the first group share 25-30% genetic similarity and include sialoadhesin, CD22, MAG, and Siglec-15, which are common in all mammals. The second group counts Siglecs related to CD33, with a genetic similarity of 50-99%, known for their rapid evolution. Despite their genetic differences, all Siglecs share a similar architecture and bind sialylated epitopes through a conserved Arg residue.
Within this context, MAG (2nd group), Siglec-6 (1st group), and Siglec-11 (2nd group), represent a bit of a puzzling case, as mutation of the conserved sialic acid-binding Arg does not affect binding affinity. In this work we used molecular dynamics (MD) simulations to further investigate the matter by rebuilding an atomistic 3D model of the complex between Siglec-6 and GM1/2 epitopes embedded in a lipid bilayer. Our results show that recognition and binding of Siglec-6 to GM1/2 involves not only the evolutionarily conserved salt bridge between Arg122 and the GM1/2 sialic acid, but also a direct contact between the protein and the bilayer through insertion of the Trp127 sidechain and by a low-specificity electrostatic adhesion mediated by different Lys residues located on the Siglec-6 binding domain. This finding was validated by binding assays, which showed that mutations in W127 and K126 abrogate binding.

This molecular-level insight suggests that Siglecs may have evolved to select for specific epitopes by uniquely adapting their structure to complement the environment where these epitopes are found. As a term of comparison, we show the results of our preliminary analysis of Siglec-10, which binds different ganglioside epitopes (GT1B) to Siglec-6, as well as secreted sialylated glycans.

(P12) How does *O*-glycosylation drive topological inversion and cell surface expression of calnexin?

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Tumour growth and metastasis depend on tissue remodelling, a process driven by extracellular matrix (ECM) degradation[1]. Glycosylation plays a key role in regulating ECM remodelling, primarily through the activation of the GALA pathway which could relocate GalNAc Transferases (GALNTs) from the Golgi to the endoplasmic reticulum (ER) and then drive protein hyperglycosylation[2]. One of these proteins is the ER resident protein Calnexin (Cnx) essential for proper protein folding and also vital to ECM degradation in cancer cells. The importance of Cnx in tumour progression is highlighted by findings that anti-Cnx antibodies inhibit liver tumour growth and lung metastasis in breast and liver cancers[3]. Interestingly, antibodies targeting the intracellular C-terminal region of Cnx also block ECM degradation, suggesting that this portion of the protein is exposed on the cell surface. Thus, we hypothesize that *O*-glycosylation induces a topology shift in Calnexin, transitioning from an "I-type" topology to a "U-type" topology.

To explore the topological change of cell surface glycosylated Calnexin, and the mechanisms underlying this phenomenon, we attempted to detect the extracellularly exposed C-terminus of Calnexin through IF and WB experiments. HaloTag (HT) was inserted at the C-terminus of Calnexin as a marker for the Calnexin C-terminal (Cnx-HT), while HaloTag can be recognized by its specific HaloTag ligands (HL). The pancreatic ductal adenocarcinoma cell line, KPC, was used as a cellular model. GALA levels were controlled by expressing an ER-localized GALNT1 under the control of a doxycycline (DOX) inducible promoter (ER-G1) to simulate high and low GALA conditions. To eliminate interference from endogenous Cnx, we knocked out Cnx and then expressed Cnx-HaloTag or HaloTag only as a control. The signal intensity from HL was compared to indicate changes in the membrane topology of Calnexin after glycosylation.

We investigated the presence of ‘U-type’ topology of Calnexin by using C-terminal HaloTag and the cell impermeant ligand HL-Alexa Fluor 660. Through the results, we can clearly say that induction of GALA (ER-G1) increased signal from the cell-impermeant ligand, which was not observed in WT, non-induced cells or by expression of HaloTag alone, suggesting an increase in ‘U-type’ calnexin at the cell surface and a link between this topological change and *O*-glycosylation. The results of different incubation times with HL-Alexa Fluor 660 showed that an increase of the signal from HL-Alexa Fluor 660 over time and transitioned from the cell membrane to the cell interior in high GALA cells, which was barely seen in other control groups. Overall, these results indicate that: the ‘U-type’ topology of Cnx exists on the cell surface, and *O*-glycosylation may drive Cnx’s topologcial change and translocation.

In conclusion, after *O*-glycosylation, Calnexin undergoes a significant change in its topology, with both the N-terminal and C-terminal domains localized outside the cell membrane. We provide preliminary evidence that the topology change of glycosylated Calnexin occurs, with the ‘U-type’ Cnx present on the cell surface. This surprising and unique topological change of glycosylated Cnx indicates a structural alteration that may be key to its escape to the cell surface. Studying this phenomenon is crucial for understanding its role in tumour growth and metastasis and exploring new therapeutic avenues targeting glycosylated Cnx. It will provide new insights into the role of the GALA pathway and how hyperglycosylation affects the structure-functional relationship of glycoproteins. This finding could also offer a new perspective and therapeutic direction for understanding tumour cell metastasis.

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(P13) Deciphering the Glycome of HER2-Positive Gastric Cancer: Towards Biological Cues and Therapeutic Implications

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The humanized monoclonal antibody trastuzumab (TTZ) remains the gold standard therapeutic modality for advanced unresectable gastric cancer (GC) with overexpression of the Human Epidermal Growth Factor Receptor 2 (HER2) oncogenic receptor tyrosine kinase. Unfortunately, the majority of treated patients experience modest and short-lived clinical benefits from TTZ due to the widespread emergence of both innate and acquired molecular resistance. Despite the extracellular domain (ECD) of HER2 undergoing extensive *N*-linked post-translational glycosylation, the precise molecular mechanisms through which cancer-associated glycan structures actively tune HER2 oncogenicity and underpin the acquisition of TTZ molecular resistance in GC remain elusive[1]. Therefore, we aimed to dissect the mechanistic role played by the cellular and HER2-specific glycome in driving GC cell malignancy, in particular the acquisition of TTZ molecular resistance, towards novel predictive and stratifying biomarkers and therapeutic targets.

Using a multi-omics approach and a comprehensive toolbox including: HER2-positive GC clinical samples[2]; CRISPR/Cas9-glycoengineered *ERBB2*-amplified GC cell lines depicting finely tuned glycosylation capacities and well-defined cell surface glycophenotypes; plant-derived recombinant HER2 ECDs produced in glycoengineered *Nicotiana benthamiana*, equipped with selected human glycosylation pathways, carrying site-specific human glycomic signatures; and TTZ-sensitive and -resistant GC cells, we have investigated the complex regulatory network between the dynamic cellular glycome, HER2-mediated oncogenic signalling and the sensitivity of HER2-addicted GC cells to TTZ-induced cytotoxicity.

Through mass spectrometry-based glycomics and glycoproteomics, we have comprehensively mapped the site-specific glycomic profile of the HER2 ECD, and identified two glycosylation sites, confined to the HER2 ECD TTZ-binding subdomain, as carriers of complex *N*-glycan species terminally modified by the Golgi-residing ST6Gal1 sialyltransferase[3,4]. ST6Gal1-mediated sialylation of HER2 actively regulates receptor dimerization and membrane turnover rate and attenuates TTZ cytotoxicity in HER2-driven GC cells. Using cancer cell- and plant-derived HER2 carrying pre-determined glycosylation profiles, we have disclosed, through surface plasmon resonance (SPR), that specific HER2 glycoforms bear differential binding affinity to clinical grade TTZ, which can, in turn, underlie differential sensitivity to TTZ. Moreover, TTZ-resistant cells exhibited an extensive remodelling of their cell surface glycome and glycan-related transcriptome, highlighting the molecular role played by cancer-associated glycans in the acquisition of TTZ resistance.

Our results pave the way for the identification of glycan-sensitive biomarkers for GC patient stratification, and the development of novel therapeutic agents capable of overcoming glycan-mediated molecular resistance.

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(P14) Sensing sialic acids with graphite-immobilised electrochemical probes

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Sialic acids (Sias) are carbohydrates found on the surface of cells or in bodily fluids. They play important roles in human physiology, and altered levels can indicate multiple disorders. Therefore, the design of new analytical methodologies for this family of monosaccharides may be useful for disease treatment and diagnosis[1]. *N*-acetyl-D-neuraminic acid (Neu5Ac) is the most common Sia naturally found in healthy humans, and it is found free in some solutions such as the cerebrospinal fluid of people with pyogenic meningitis[1]. Boronic acids are well-known carbohydrate receptors which form pH-dependent covalent interactions with diol-containing target molecules, leading to boronate ester species; at acidic pH values, they bind α-hydroxy acids, which are present in Sias but are not present on many other monosaccharides. Electrochemical sensing of Sias using commercially available electroactive boronic acids is limited by their structural simplicity and can result in interference from α-hydroxy acids such as lactic acid, but it can be easily implemented compared to other methods and may avoid interference from neutral saccharides[2].

We have found that immobilising electroactive boronic acid-containing compounds in a carbon paste matrix responds to Neu5Ac while reducing interference from lactic acid. This may be due to CH-π interactions with the graphite of the carbon paste, which improves selectivity. Carbon paste electrodes with encapsulated redox-active chemoreceptors enable direct sensing from buffered clinical solutions without the need for organic solvents. Thus, in this work, we present a sensor based on ferroceneboronic acid immobilised in a graphite/paraffin carbon paste matrix, with performance evaluated relative to parallel homogeneous binding studies. Qualitative and quantitative Neu5Ac interactions were examined by monitoring the altered redox properties of the chemoreceptor. The binding properties of this probe establish it as a portable, rapid and selective method of detecting Neu5Ac in the presence of other monosaccharides.

In addition to this, a series of novel electroactive ferrocene phenylboronic acids were synthesised and characterised (including X-ray crystallography). The anionic Neu5Ac (p*K*a = 2.6)[1], which includes an α-hydroxy acid group, was once more employed for selectivity purposes, providing rationale for probe design and binding conditions. The redox properties of the synthetic probes were established via cyclic voltammetry and differential pulse voltammetry, which led to the most promising compound for Neu5Ac recognition. Solution phase studies confirmed that Fc-LC apparently recognises Neu5Ac with reduced interference from lactic acid. Follow-on steps will involve incorporation of this novel synthetic probe into the carbon paste matrix as an electrochemical sensor.

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(P15) Targeting the Immunosuppressive Tumor Microenvironment in Lung Cancer with Anti-Bacterial Capsular Polysaccharide Antibodies

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Over the past decade, various large-scale studies in cancer patient tissues revealed the presence of specific bacteria within and around cancer cells in the tumor microenvironment (TME). Interestingly, TME-residing bacteria directly influence tumor initiation, progression, response to therapy, and patient prognosis. In lung cancer, dysbiosis of the lower airway flora, enrichment of the lung microbiome with oral bacteria, and reduced microbial diversity are associated with chronic lung inflammation and increased risk of cancer.

Streptococcus pneumoniae is an opportunistic pathogen that colonizes the mucosal surfaces of the human upper respiratory tract. The bacteria are significantly enriched in the lower airway flora of lung cancer patients and correlate with increased cancer risk and secretion of toxins associated with cancer development.

The bacterial capsular polysaccharides (CPS) layer is a crucial virulence factor that directly engages with the host immune system and protects the bacteria from immune system effector cells. The high diversity, structural complexity, and thickness of the CPS layer define more than 100 known S. pneumoniae serotypes and mediate their immune evasion and ability to colonize the host. The contribution of S. pneumoniae different serotypes to lung cancer tumorigenesis or the different distribution and dispersion of individual serotypes at the TME and between cancer patients still awaits discovery.

Using well-defined synthetic S. pneumoniae serotype 14 (SP14), we generated a unique single-domain antibody ("Nanobody" or Nb) that binds specifically to SP14. Performing immunohistochemical analyses with the Nbs against patient-derived lung cancer tissues enabled a unique identification of SP14 at the TME and inside tumor-associated macrophages (TAMs), possibly correlating SP14 levels with TAMs distribution. We then confirmed the presence of SP14 solely in lung cancer and not healthy tissues by using an environmental scanning electron microscope (ESEM) of patient samples. Current work elucidates SP14's interactions with TAMs in lung cancer and its contribution to pro-tumorigenic conditions. In addition, we test the Nbs, independently and as bi-specific engagers, as novel lung cancer theranostic tools in vitro and in a xenograft mouse model.

(P16) Hyper *O*-glycosylation drives cell competition in liver tumours

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The interface between normal and transformed epithelial cells at the tumour edge remains poorly understood. In solid tissues, cancer cells must outcompete normal cells for space, but the mechanisms behind this process are not well known. Cell competition, observed in multicellular organisms, is thought to eliminate defective cells and may act as an "epithelial defense against cancer". However, cancer cells function as "super-competitors," which allow them to form tumour nodules.

The molecular processes underlying cell competition implicate various known pathways and oncogenes, such as the Hippo and/or Wnt pathways, the Myc, Src and EGF-R oncogenes and others[1]. However, little is known about the cell surface molecules involved in cell competition. Cell surface proteins are in their vast majority glycoproteins, carrying various *N*- and *O*-glycans. Cancer is associated with massive changes of glycosylation. In particular, O-glycosylation is frequently altered, with an increase in *O*-glycans sialylation and especially an increase in the Tn glycan[2].

Cell surface protein glycosylation occurs in the secretory pathway, a complex and highly compartmentalized system composed of two main organelles: the ER and the Golgi apparatus. We have found that GALNTs, normally localised in the Golgi, are re-located in the ER upon activation of signalling molecules such as the Src and EGF-R kinases. This relocation results in increased O-glycosylation and a marked increase in cellular Tn levels[3]. We nicknamed GALA (GALNTs Activation) pathway, this highly regulated activation of *O*-glycosylation. We found that GALA regulates cell adhesion and migration.

We discovered that when the GALA pathway is initiated and gradually intensifies in tumour cells, tumour growth accelerates compared to tumours with lower levels of GALA. In contrast, depleting both GALNT1 and 2 significantly reduces the development of liver tumours. In a converse experiment, when GALA pathway was activated in the whole liver, the liver was protected from tumour growth. So, GALA, strongly stimulates growth when activated only in tumour cells but slows tumour growth when expressed in neighbouring, non-transformed cells. These data strongly suggest that GALA mediates a form of cell competition essential for tumour growth: high GALA out-compete low GALA cells.

To investigate the nature of this competition and identify the molecular players involved, we sought to replicate this phenomenon in an in vitro setting. We initiated coculture experiments with high GALA and low GALA cells to determine whether low GALA cells exhibit an increased rate of apoptosis when cocultured with high GALA cells compared to conditions where they are cocultured with the same cells with equal level of GALA. We evidenced the distinguishable interactions between high GALA and low GALA cells resulting in apoptosis in low GALA cells in timelapse microscopy. Further quantification of these timelapse videos revealed that high GALA cells tend to interact repeatedly with low GALA cells.

To enable high-throughput quantification of the increased cell death in low GALA cells, we developed an endpoint assay from image acquisition to image analysis to measure the percentage of apoptosis in low GALA cells in coculture with high GALA cells, compared to conditions where glycosylation levels are equal in both cell populations.

Our findings showed that apoptosis in low GALA cells increases when they are exposed to high GALA cells, as opposed to when they are cocultured with cells with the same degree of glycosylation. Further research is required to identify the apoptotic pathway in low GALA cells and the molecular participants involved in these interactions.

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(P17) Flavonoid-mediated inhibition of NLRP3 inflammasome activation: anti-inflammatory insights from C*apparis cartilaginea decne*

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Inflammation is a vital immune response, with the NLRP3 inflammasome playing a key role in mediating inflammatory activation[1,2]. Dysregulation of NLRP3 is associated with diseases such as gout and type 2 diabetes, making it a promising therapeutic target[2,3]. Flavonoids, abundant in plant sources, have garnered attention for their anti-inflammatory, antioxidant, and anti-apoptotic properties, offering potential for disease prevention and protein activity modulation[1,4,5].

This study investigates the inhibitory potential of flavonoids isolated from the tea leaves of *Capparis cartilaginea decne*, a plant widely consumed for its health benefits and cultivated in the Al-Taif region of Saudi Arabia[6]. The identified compounds include quercetin 3-O-(2G-rhamnosylrutinoside) (Manghaslin), quercetin 3-O-neohesperidoside (Q3-Neo), quercetin 3-O-rutinoside (Rutin), kaempferol 3-O-(2G-rhamnosylrutinoside) (Clitorin), kaempferol 3-O-neohesperidoside (K3-Neo), kaempferol 3-O-rutinoside (Nicotiflorin), and isorhamnetin 3-O-rutinoside (Narcissin). *In silico* analysis, including molecular docking and molecular dynamics (MD) simulations, as well as *in vitro* assays such as ELISA and Western blotting, were conducted to explore the flavonoids' potential as NLRP3 inhibitors. Among the tested flavonoids, all demonstrated favourable interaction profiles with the NLRP3 active site, with quercetin derivatives, particularly Q3-Neo and Manghaslin, exhibiting the strongest inhibitory interactions *in silico* through hydrogen bonding with key amino acid residues. MD simulations further confirmed these interactions, showing stable and robust binding dynamics. Other flavonoids, including Narcissin and Nicotiflorin, also exhibited significant inhibitory potential, while Clitorin and K3-Neo displayed weaker binding dynamics. The interactions between the hydroxyl groups of the ligands and the carboxylate side chains of Glu629 and Arg578, observed in this study, align with previous findings that highlight these interactions as crucial for effective inhibitor-receptor binding[7,8].

These computational findings were corroborated by *in vitro* assays, which demonstrated significant inhibition of NLRP3 inflammasome activation. Rutin (40.6% ± 4.0%), Manghaslin (38.9% ± 6.31%), and Q3-Neo (29.8% ± 8.2%) exhibited the highest inhibitory effects among the quercetin-based flavonoids. Nicotiflorin, a kaempferol-based flavonoid, also showed promising inhibition (32.25% ± 4.22%). These results reinforce the potential of quercetin-based flavonoids as selective NLRP3 inhibitors and support their development as lead compounds for anti-inflammatory therapeutics[9,10,11].

In conclusion, this study provides novel insights into the structural modulation of NLRP3 by flavonoids and highlights the therapeutic potential of quercetin-based compounds, particularly Q3-Neo and Manghaslin, in targeting inflammation-related diseases. These findings pave the way for further investigations into their mechanism of action and potential clinical applications.

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(P18) A2M *N*-glycosylation is essential for its protease inhibition and anti-inflammatory functions in chronic liver disease

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Introduction: Current worldwide estimates show that about 1.5 billion people are living with chronic liver disease (CLD), with a mortality rate of 1.6-2 million deaths yearly. The progression of CLD is characterised by dysfunctional immune responses and systemic inflammation. Alpha-2-macroglobulin (A2M) is an acute phase glycoprotein involved in the regulation of systemic inflammation and is produced by the liver. It's generally present in serum as a 720kDa monomer with 32 possible *N*-glycosylation sites. Despite being so abundant, there is a paucity of data on the functions of A2M and the role of *N*-glycosylation in regulating these functions. While it is generally accepted that its main function is the inhibition of protease activity, there is also data to suggest that it may act on other molecules, such as cytokines and growth factors, as an inhibitor or a transporter.

Hypothesis: The aim of this study is to determine if A2M *N*-glycosylation is relevant to its functions and if altered *N*-glycosylation in CLD influences its functional properties.

Methods: Functional assays to assess protease-inhibitor activity and interleukin (IL)-1β signalling were established using the Pierce protease assay and THP-1 Dual reporter cells. To assess the importance on A2M *N*-glycosylation we utilised PNGase digestions of A2M to remove the outer *N*-glycans on A2M. The THP-1 dual reporter model analysed the impact of A2M *N*-glycans on NF-κβ induction. Optimisation of *N*-glycosylation profiling of A2M by Hydrophilic Interaction Liquid Chromatography Ultra Performance Liquid Chromatography (HILIC-UPLC) was performed using commercially purified human serum A2M. We then isolated A2M from the serum using a combination of IgG and albumin depletion and size exclusion using a Cytiva CaptoCore 700 column, and profiled the *N*-glycosylation in clinical samples from control and CLD patients using UPLC.

Results: A2M *N*-glycosylation profiling using our method has over 44 unique glycan species. Previously the most identified was 27 species (Arnold *et. al*., 2006). Removal of A2M *N*-glycans caused it to lose ~70% of its trypsin inhibitory capacity. We also observed that A2M can completely abolish IL-1β's ability to activate NF-κβ signalling ($p=0.0004$). Removal of the *N*-glycans from native A2M resulted in a significant reduction ($p=0.0082$) in its ability to inhibit IL-1β signalling, with a 33% restoration of its inhibitory ability. Finally, we observed *N*-glycosylation alteration in CLD patients in comparison to healthy controls.

Conclusion: A2M is an important molecule for controlling inflammatory processes in diseased states. Here for the first time, we have not only characterised A2M *N*-glycosylation with greater precision than has been done previously, we have also shown the importance of A2M *N*-glycosylation in the regulation of protease activity and the control of proinflammatory molecule IL-1β which is involved in the progression of CLD

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(P19) Selective Ruthenium-Based Inhibitors of Human Galectin-1 with Antitumor Potential

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Galectins are a family of β-galactoside-binding lectins involved in diverse biological processes, including tumour progression. Human galectin-1 (*h*gal-1) is implicated in cell migration, tumour immune escape, and apoptosis regulation, making it an attractive target for anticancer therapy[1-3]. Here, we present a novel class of highly selective *h*gal-1 inhibitors based on ruthenium piano-stool complexes conjugated to the *N*-acetyllactosamine and thiodigalactoside scaffolds.

The developed ruthenium-based glycomimetics exhibit nanomolar binding affinities to both human and mouse gal-1 while displaying unprecedented selectivity, with a more than 1000-fold preference for *h*gal-1 over human galectin-3 (*h*gal-3). This level of selectivity is unparalleled in the field of galectin inhibition and underscores the potential of these organometallic structures as a possible next generation of highly selective galectin inhibitors. The exceptional selectivity of these inhibitors stems from the sterically demanding organometallic moiety fitting snugly into the larger carbohydrate recognition domain (CRD) cavity of *h*gal-1, while being too bulky to occupy the smaller CRD of *h*gal-3.

Biological evaluation of these inhibitors revealed their ability to selectively reduce the viability of *h*gal-1-expressing MDA-MB-231 cancer cells at low micromolar concentrations, while showing negligible cytotoxicity toward galectin-1-null HEK-293 noncancerous cells. Additionally, the inhibitors effectively scavenge extracellular *h*gal-1 and prevent its binding to the surface of MDA-MB-231 cells. This ability to block extracellular *h*gal-1 interactions is crucial, as it mimics *in vivo* behaviour and highlights the potential of the inhibitors to disrupt *h*gal-1-mediated signalling pathways. Furthermore, these compounds inhibit *h*gal-1-induced apoptosis in Jurkat cells, reinforcing their therapeutic relevance.

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(P20) Building the next generation of biomaterials: glycan-modified polyethylene glycol

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Biomaterials are extensively employed by researchers and medical professionals in a wide array of medical applications, encompassing targeted drug delivery systems, implantation procedures, and wound dressing materials. Nevertheless, the hosts predominantly reject them, thereby constraining their potential and reducing their lifespan. It is imperative to develop biomaterials with better physiochemical properties that can surmount immune rejection and deliver complete therapeutic efficacy. One possible way to achieve this is to functionalise the surface of biomaterials with glycans to mimic the host environment better[1].

When a material comes into contact with the host tissue, a series of events occur, activating the immune system. Glycans are involved in the immune system in various ways[2]. Glycans decorate all immune receptors on the cell surface, and molecules secreted under inflammatory conditions, such as chemokines, cytokines, antibodies, and complement components, are all glycoproteins. Glycan-binding proteins (lectins) are found on the surface of immune and immunomodulatory cells. Targeting these lectin receptors through glycan ligands can help develop immune-evasive biomaterials[3].

To this end, we propose the development of a diverse library, a model polymer—linear polyethylene glycol (PEG)—functionalised with eleven distinct monosaccharides. PEG, characterised by its simplicity, finds widespread application in the fabrication of biomaterials and various other biomedical contexts[4]. The monosaccharides attach to the two terminal groups of PEG, whereas the ethylene oxide repeating units remain unreactive owing to their lack of side functional groups. This method of delivering sugar molecules offers two advantages: it prevents side reactions from the PEG carrier and increases the effective size of monosaccharides. Sugar-modified linear PEGs were synthesised in three steps: acetylation of monosaccharides, PEGylation of acetylated monosaccharides, and final deprotection to yield modified linear PEGs. PEGylation was performed using both the conventional methods and microwave-assisted synthesis, with the latter producing higher yields. Each step was characterised and confirmed using FT-IR, 1H, and 13C NMR. Integration of the peaks confirmed the bi-functionalisation of the linear PEG. MALDI-TOF and GPC confirmed molecular weight changes in the PEG-modified and thermal properties were recorded using DSC and TGA.

The cytotoxicity of different concentrations of sugar-modified PEGs was assessed in bone marrow-derived mesenchymal stem cells (MSCs) and human dermal fibroblasts (HDFs) using alamarBlue™, Quant-iT™ PicoGreen™, and live/dead assays. The results demonstrated that successful PEG saccharide modification did not induce any cytotoxicity. Analysis of the MSC secretome in the presence of PEGylated monosaccharides using a multiplex assay under inflammatory conditions is ongoing to investigate their immunomodulatory effects. In conclusion, sugar-modified PEG holds promise for the development of advanced biomaterials with enhanced host response interfacial properties.

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(P21) Development of a novel green strategy for fluorescent labelling of IgG *N*-glycans

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Sustainability in glycoanalytical methods is becoming increasingly important, especially in therapeutic glycoprotein characterisation. This study investigates nine solvents from the dipolar aprotic, non-polar, and polar protic categories in search of a more sustainable alternative solvent to acetonitrile (MeCN) for IgG *N*-glycan labelling fluorescent labelling with 6-aminoquinoline (AQC)[1]. Cyrene, *N*-methyl-2-pyrrolidone (NMP), and dimethylformamide (DMF) are dipolar aprotic solvents, while 2,2,5,5-tetramethyloxolane (TMO) and toluene are non-polar solvents, and ethanol, methanol, and ethylene glycol are polar protic solvents[2,3]. Each solvent has been assessed for compatibility, efficiency, and reproducibility in glycan labelling procedures.

One major discovery of this investigation was the identification of TMO and Cyrene as suitable green substitutes to replace MeCN in our IgG *N*-glycan quantitation workflows[4,5]. Both solvents exhibited excellent good reproducibility, preserving neutral and sialylated glycan profiles while providing analytical performance comparable to MeCN-based conditions. The new approach integrates these solvents into glycan labelling techniques while maintaining sensitivity and precision, as confirmed by UPLC analysis.

To improve glycoanalytical accuracy, an internal standard was also developed that allows for the quantification of individual glycans and peak assignment in the picomolar range using UPLC approaches. This internal standard also provides qualitative fluorescence data, revealing how different solvents influence the fluorescence of AQC. By assessing fluorescence variations, this approach ensures the selection of an optimal solvent that maintains analytical performance while offering a greener alternative.

This study is a significant step forward in sustainable glycobiology, harmonising with the global shift towards environmentally responsible scientific procedures. By replacing conventional solvents with greener alternatives and incorporating an internal standard for enhanced quantification and fluorescence assessment, this study minimises chemical hazards while advancing biopharmaceutical analytics. With optimisation nearing completion, our findings offer a scalable and effective option for long-term glycoanalysis, promoting safer laboratory practices while keeping high-performance analytical capabilities.

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(P22) Recent Updates in the Glyco@Expasy Suite

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The Glyco@Expasy suite of software and databases serves as a central hub for glycobioinformatics, providing researchers with tools to analyse and interpret glycan-related data. Recent updates to this suite have significantly expanded its capabilities, particularly in GlyConnect, a comprehensive glycan structure and protein glycosylation database, and its associated services based on so-called semantic web technologies. These advancements enhance data accessibility, interoperability, and analytical power, catering to the growing demands of glycobiology research.

GlyConnect has been significantly refined to offer improved data integration, visualization, and retrieval functionalities. The latest update includes a more extensive collection of glycan structures, glycosylation sites, and protein associations curated from high-throughput glycoproteomics studies. New visualization tools have been incorporated, allowing users to explore glycan structures interactively, analyse glycosylation patterns on proteins, and cross-reference with other molecular data sources. Furthermore, improved search algorithms and user-friendly interfaces facilitate efficient querying and interpretation of complex glycosylation datasets.

In addition to these key advancements, the Glyco@Expasy suite has improved its documentation and support features, ensuring that researchers can effectively navigate and utilize its resources. Tutorials, case studies, and interactive guides have been updated to help users maximize the potential of GlyConnect and data integration services.

These recent updates position Glyco@Expasy as a more robust and interconnected platform for glycomics research, facilitating the exploration of glycan structures, protein glycosylation, and their broader biological implications. Future developments will continue to focus on expanding database coverage, refining analytical capabilities, and integrating emerging glycoproteomics technologies. By leveraging the power of semantic web technologies and enhanced data curation, Glyco@Expasy remains at the forefront of glycoinformatics, supporting the advancement of glycobiology in biomedical research and therapeutic discovery.

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(P23) High sensitivity identification of *N*-linked glycopeptides in human plasma using alternative fragmentation

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Main body: Protein glycosylation is a critical post-translational modification (PTM) that affects protein structure, stability, and function. Mass spectrometer (MS) instrument sensitivity is a significant limitation when analyzing glycopeptides. Due to the high degree of heterogeneity of glycan structures, the abundance of each glycoform is relatively low. The challenges associated with low glycopeptide abundances can be overcome with strategies like enrichment of glycopeptides, a lower flow rate LC separation regime (i.e., nanoflow LC separation), and utilization of MS systems with high MS/MS sensitivity for glycopeptide detection and characterisation. In addition, alternative MS/MS fragmentation techniques, such as electron-activated dissociation (EAD), complement traditional collision-induced dissociation (CID) for the complete characterisation of glycopeptides

High-abundance proteins were depleted from human plasma using Top14 Depletion spin columns (Thermo Fisher). After digestion of the remaining proteins with trypsin digestion, glycopeptides were enriched using BioSPE GlycanClean kits (Affinisep). Nanoflow separation was done with a Waters M-Class HPLC system using trap-elute nanoflow (300 nL/min) with a 60-minute reverse-phase gradient on a 0.075 x 250 mm C18 column. And data-dependent acquisition was performed using a novel quadrupole time-of-flight (QTOF) mass spectrometer, using both CID and EAD MS/MS fragmentation. Data were processed using PEAKS GlycanFinder software (BSI Inc).

The use of simplified commercial sample preparation strategies resulted in the successful depletion of high-abundance plasma proteins and the highly efficient enrichment of tryptic glycopeptides. Nanoflow separation enabled a greater depth of identification compared to higher flow rate LC regimes, particularly with limited amounts of sample. Using a combination of EAD fragmentation and high-sensitivity MS/MS on a novel QTOF platform, >1500 glycopeptides were identified in the equivalent of 50 ng protein from depleted human plasma, representing a significant increase over previously reported results. The high MS/MS signal intensity allowed for the efficient identification of glycopeptides from injections of less sample. Using PEAKS GlycanFinder software to process EAD MS/MS data, the glycopeptide backbones were efficiently sequenced using the resulting c and z' fragment ions, which also retained the intact glycan modifications. From the EAD MS/MS results, glycan structures and exact glycan PTM positions were determined. This contrasts with CID MS/MS data, which generated predominantly oxonium and internal fragment ions.

(P24) Glycogenomics: mass spectrometry driven insights into sialyltransferase-specificities across multiple glycan classes

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Glycosyltransferases are essential enzymes that catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, playing a critical role in the biosynthesis of functional glycan motifs—a process vital for proper cellular function and signalling. The six-membered ST3GAL family of sialyltransferases catalyses the transfer of sialic acid in an α2,3-linkage to galactose residues on the outermost glycan chain epitopes. The dysregulation of sialyltransferase activity has been implicated in numerous pathological processes, including cancer metastasis, where aberrant sialylation can enhance cell migration and immune evasion. ST3GAL expression is known to be abundant in certain tissue types, including the liver and brain and α2,3-linked sialylation plays a direct role in mediating interactions between siglecs and selectins, and their glycoconjugate ligands[1], highlighting the diverse roles of sialylation in both normal physiology and disease progression.

While the enzyme ST3GAL1 exhibits a well-defined specificity for the sialylation of core-1 *O*-glycans, and ST3GAL5 is known as the glycolipid GM3 synthase, the remaining members of the ST3GAL-transferase family have more ambiguous specificities raising important questions about how substrate selection is regulated within the ST3GAL family, especially in complex cellular systems. Most of our knowledge on sialyltransferase substrates comes from *in vitro* testing, the enzyme specificities in cellular systems have not been systematically addressed.

We employed a strategy that enables sequential glycomics from the same sample to systematically analyse the role of the subfamily members of ST3GAL across *N*-, *O*-, and GSL glycans. Using CRISPR/Cas9-mediated gene knockouts, a series of glycoengineered human keratinocytes (N/TERT-1) were generated deficient in specific sialyltransferases genes or a combination thereof, enabling us to study their individual effects on the glycan biosynthesis. For the in-depth analysis of glycan isomers, we employed both 2-aminobenzamide labelling of the glycans (*N*- and *O*-linked) to enable efficient C18 LC-MS/MS analysis[2] and PGC-LC-MS/MS analysis of reduced GSL glycans, for detailed structural characterization.

These analyses showed the expected effects on *O*-glycans and GSL glycans for ST3GAL1 and ST3GAL5 KOs, respectively. Interestingly, while the KO of ST3GAL1 and ST3GAL2 individually did not completely ablate *O*-glycan core 1 sialylation, the combined KO did. A similar effect was observed for the sialylation of *N*-glycans by ST3GAL4 and ST3GAL6, where only the combined KO resulted in maximum ablation of the epitope. In contrast, for *O*-glycans, LacNAc α2,3-sialylation was affected solely by the KO of ST3GAL4.

In conclusion, our work advances the understanding of sialyltransferase specificity in complex cellular models, revealing specific effects of these enzymes on glycoconjugate biosynthesis that differ from, or are more defined than, what was previously described. This new knowledge will aid in unraveling the roles of glycosyltransferases in human diseases.

(P25) High-throughput LC-MS method for site-specific *N*-glycosylation profiling of human fibrinogen

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Fibrinogen is a key plasma glycoprotein involved in coagulation and inflammation, with its glycosylation playing an important role in modulating fibrin structure and function. However, site-specific analysis of fibrinogen *N*-glycosylation in clinical samples has been hindered by analytical challenges, including the need for costly antibody-based enrichment, limited sensitivity in complex plasma matrices, and insufficient throughput for large-scale studies. To overcome these limitations, we developed a high-throughput LC-MS workflow for site-specific fibrinogen *N*-glycosylation analysis, employing a cost-effective ethanol precipitation method for direct enrichment from plasma samples. This workflow enables robust and reproducible glycopeptide analysis, achieving intra- and inter-plate relative standard deviations of 5% and 12%, respectively. We validated the method by assessing intraindividual temporal stability of fibrinogen glycosylation in healthy individuals, confirming consistent glycosylation patterns over time.

As a pilot clinical application, we analysed fibrinogen *N*-glycosylation in a cohort of 181 atrial fibrillation (AF) patients and 52 healthy controls. We identified three γ-chain glycoforms significantly associated with AF, with the most notable alteration being increased levels of the asialylated N4H5 glycoforms, previously linked to enhanced fibrin bundle formation and a prothrombotic state. Additionally, fibrinogen sialylation showed strong associations with cardiovascular risk factors, including triglycerides, BMI, and glucose levels. Longitudinal follow-up of 108 AF patients six months post-catheter ablation demonstrated stability of the AF-associated glycosylation profile.

Our findings establish fibrinogen glycosylation as a promising biomarker for cardiovascular conditions and highlight the utility of the developed LC-MS method for translational research and large-scale investigations into glycosylation-driven disease mechanisms.

(P26) Fucosylation and Sialylation Exhibit Distinct Expression in Mature and Tolerogenic Human Monocyte-Derived Dendritic Cells

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Foreign Body Response (FBR) poses a significant challenge to the long-term success of implants, often leading to deleterious outcomes[1-3]. To mitigate this, it is critical to promote a tolerogenic and wound-healing immune environment around implants. Despite the progress in biomaterials and immunomodulatory approaches, a limited number of clinically approved combination products, biologics, and materials have exhibited a significant and manageable ability to influence the outcome of implanted devices [4-7].

Dendritic cells (DCs) are central regulators of immune responses and act as a bridge between innate and adaptive immunity[8,9]. Among the various molecular mechanisms governing DC function, glycosylation (the most common post-translational modification) plays a crucial role in immune regulation[10]. Glycosylation influences protein folding, intracellular trafficking, stability, and protection from proteolysis[11-14]. Certain glycans on the DC surface serve as key regulators of immune signalling, shaping interactions with effector cells and influencing whether DCs drive immune activation or tolerance[15]. Previous studies using Bone Marrow-Derived Dendritic Cells (BMDCs) from Dark Agouti rats and human Monocyte-Derived Dendritic Cells (moDCs) suggested that DC surface sialylation dynamically changes depending on environmental cues and functional state, leading to distinct immune signalling outcomes[16-18]. Global glycosylation patterns in dendritic cells (DCs) and their modifications during polarization in response to various stimuli remain largely unexplored.

We hypothesize that DCs actively remodel their surface glycosylation in response to distinct environmental signals, and this differential glycosylation landscape modulates the biomaterial-host immune responses. Using human moDCs, we identified distinct glycosylation signatures associated with pro-inflammatory mature DCs (mDCs) and anti-inflammatory tolerogenic DCs (tDCs).

Our results demonstrate that α2,6-sialylation (SNA, WGA) increases upon DC maturation due to elevated ST6GAL1 expression during monocyte differentiation[19,20]. Additionally, we observed a significant reduction in DC-SIGN expression in mDCs, corresponding to their decreased endocytic activity, which is consistent with reports that ecto-sialyltransferases suppress endocytosis in moDCs[21]. Importantly, we also show for the first time that IL-10-induced tDCs exhibit downregulated fucosylation. This finding aligns with previous studies demonstrating that inhibiting fucosylation shifts macrophages towards an anti-inflammatory (M2) phenotype[22] and that IL-10-producing CD4+ T cells negatively regulate epithelial fucosylation[23].

Together, these results highlight the crucial roles of sialylation and fucosylation in DC biology and suggest that glycosylation plays a key role in shaping immune responses. By elucidating the role of DC glycosylation in immune regulation, this study provides valuable insights for designing stable immunomodulatory approaches that have the potential to mitigate the fibrotic encapsulation of implantable medical devices. Advancing this approach could significantly improve patient outcomes and revolutionize the field of biomaterials and medical device engineering.

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(P27) Glycoprofiling of extracellular vesicles by lectin-based methods

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Extracellular vesicles (EVs), including exosomes, are small, membrane-bound particles released by cells in both healthy and diseased states. They play a vital role in cell communication, immune response, gene regulation, metabolism, and wound healing. Their surface glycosylation patterns influence biological recognition[1,2].

In this study, we will work on a comprehensive glycoprofiling of EVs/exosomes based on affinity lectin-using techniques such as surface plasmon resonance (SPR), a sensitive, label-free optical technique that enables real-time analysis of biomolecular interactions, and lectin-based microarray, a high-throughput method with fluorescent labelling. We are focused on bladder cancer-related EVs. EVs/exosomes will be isolated via ultracentrifugation and characterized using Nanoparticle Tracking Analysis (NTA) to confirm size distribution and concentration while preserving native glycan integrity. For glycan analysis, we will test several strategies, such as the direct immobilization of EVs on sensorchip and microarray biochip substrates, the immobilization of EVs using antibody-functionalized chips, and lectin-coated chips to identify distinct glycan motifs.

This approach aims to capture dynamic glycan-protein interactions while maintaining exosome structural fidelity in several ways, to select the most suitable, and to use the data obtained from them for further processing using bioinformatics tools. As is well known, many diseases, including various cancers, are associated with altered glycosylation patterns that influence disease progression. By comparing EVs glycan profiles between healthy and diseased individuals, this study seeks to identify disease-specific glycosignatures with potential diagnostic utility. The SPR-based platform offers significant advantages for glycoprofiling, including high sensitivity and the ability to monitor interactions without labelling, while the microarray platform enables high-throughput analysis.

The findings may contribute to the development of non-invasive biomarkers of bladder cancer and targeted therapies, with broader implications for precision medicine in cancer and other disorders.

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(P28) Multi-machine learning algorithms and RSM method constructing intervertebral disc cells glycosylation model in vitro

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Post-translational modifications (PTMs) are chemical alterations in proteins that occur after the completion of protein translation. These modifications frequently affect protein function by regulating protein stability, localisation, and interactions with other molecules[1]. Glycosylation, a pivotal PTM, is a process by which carbohydrates are enzymatically attached to proteins or lipids. It plays a critical role in protein folding and stability, subcellular localisation, and glycoprotein functionality[2]. Protein glycosylation has emerged as a major area of research interest because of its diverse roles in cellular activities and its growing recognition as a pivotal factor in inflammation and disease.

In our prior studies with intervertebral disc degeneration (IVDD) glycome in human intervertebral disc degeneration, we have seen the comprehensively N-glycome of the human disc with spatial and temporal resolution[3]. The regulation of glycosylation in IVDD is closely associated with inflammatory pathways[3]. However, the current understanding of specific structure-function relationships and the role of specific glycans in IVDD remains incomplete[4]. This underscores the potential of glycosylation as a target for in the treatment of IVDD by regulating cytokines and glycosylation. Moreover, there is an absence of an in vitro IVDD glycosylation model to study glycosylation profiles[5]. This is predicated on prior IVDD glycosylation obtained from patients with IVDD.

To address this need, we developed 15 machine learning algorithms and corresponding experimental approaches to optimise an IVDD glycosylation model. This model encompasses parameters such as pH, glucose concentration, oxygen concentration, cytokines, and osmolarity of the medium to quantify lectin staining (ConA, UEA, and SNA) and UPLC. The optimised IVDD model was then subjected to in vitro analysis to identify key factors influencing its performance. The results of this study demonstrated that although different osmolarities can influence the SNA of intervertebral disc cells, this effect was not statistically significant ($P < 0.05$). In this study, we developed an in vitro glycosylation model for IVDD using a cross-modelling approach.

This model provides a suitable in vitro framework for a cell-free approach to construct interventional glycosylation for IVDD treatment. Furthermore, we observed the metabolic and cellular phenotypes of the optimised IVDD in vitro glycosylation model.

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(P29) Endometriosis specific vaginal microbiota links to urine and serum *N*-glycome

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Endometriosis is a chronic systemic disease in which tissue similar to uterine lining grows outside the uterus. This condition affects approximately 10% of reproductive-aged women worldwide. Its aetiology is poorly understood, and no long-term cure is available. The development and persistence of the disease depend on several coexisting factors. The vaginal microbiome is one of these factors, but its role in endometriosis and its systemic involvement is not fully understood.

Here we investigated the vaginal microbiota, serum and urine glycome and immunoglobulin G glycosylation in endometriosis patients in comparison with controls - infertility patients with similar complaints but no clinical evidence of endometriosis. We identified an endometriosis-specific vaginal microbiota, that was not present in controls. Endometriosis patients exhibited a loss of the dominant *Lactobacillus* species, *Lactobacillus iners*, and presented increased bacterial diversity, including species such as *Anaerococcus senegalensis, Anaerococcus octavius, Prevotella jejuni* and *Porphyromonas bennonis*. The levels of trigalactosylated and triantennary serum glycans, as well as core fucosylated mono-antennary glycans from urine IgG correlated with the levels of *A. senegalensis* in endometriosis patients. Although urine glycans did not significantly differ in endometriosis, they contained four novel sulfated glycans distinct from serum IgG, indicating functional relevance.

Our findings contribute to a deeper understanding of the relationships between the vaginal microbiota and the serum and urine glycome in endometriosis. The link between the local vaginal microbial environment and the systemic nature of endometriosis is evident in the altered urine and serum glycans which correlate with vaginal microbial species. Further investigation of microbiota identification and diversity typing in conjunction with host and clinical factors, will refine the diagnosis and understanding of the aetiology of this enigmatic chronic disease. Further functional studies based on these findings are warranted.

(P30) Simulated digestions of milk oligosaccharides reveal unexpected microbial syntrophies

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The development of a stable human gut microbiota occurs within the first year of life. Many open questions remain about how microfloral species are influenced by the composition of milk, in particular its content of human milk oligosaccharides (HMOs). We investigated the effect of the human HMO glycome on bacterial symbiosis and competition, based on the glycoside hydrolase (GH) enzyme activities known to be present in microbial species. To that end, a list of all bacterial species catalysing glycoside hydrolase activities (EC 3.2.1.-) was extracted from UniProt[1] and cross-referenced with BRENDA[2] to obtain a set of taxonomic lineages and CAZy-family data[3]. A set of 13 documented enzyme activities was selected and modelled within an enzyme simulator according to a method described previously in the context of HMO and *O*-glycan biosynthesis[4,5].

A diverse population of experimentally observed HMOs was fed to the simulator, and the enzymes matching specific bacterial species were recorded, based on their appearance of individual enzymes in the UniProt dataset. The potential of microbes to digest either HMOs or the surface glycans of the intestinal mucosa, and the possibility of cross-feeding and syntrophy was investigated. Pairs of bacterial species were identified that possessed complementary enzyme profiles enabling the digestion of the HMO glycome, from which potential symbioses could be inferred. Conversely, bacterial species having similar GH enzyme profiles were considered likely to be in competition for the same set of dietary HMOs within the gut of the newborn. *B. bifidum*, *B. longum* and *C. perfringens* species were predicted to have the most diverse GH activity and therefore to excel in their ability to digest these substrates, a result that was validated against the CAZy database. The expected cooperative role of Bifidobacteriales contrasts with the surprising capacities of the pathogen, *Clostridium perfringens*, which is known to play a role in necrotising enterocolitis[6].

These findings indicate that potential pathogens may associate in human gut based on their shared glycoside hydrolase digestive apparatus, and which, in the event of colonisation, might result in dysbiosis. The method described can readily be adapted, as new degradative enzymes are identified and included in the model[7].

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(P31) Nanoparticles as a platform for human plasma prefractionation

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N-glycan glycosylation changes are known to be affected in relationship to disease, and the detection of glycan changes is becoming more impactful as an early warning disease, predicting response to treatment and in patient stratification.

The high level of complexity and interplay between different intrinsic and extrinsic factors could complicate the data interpretation and loss of potential biomarkers, especially with complex samples and diseases with multiple mechanisms, risk factors and treatment regimes.

Nanoparticles (NPs) are objects that are at least 100nm in one dimension. They have been widely used in a wide range of applications in nanomedicine. However, their exploitable properties make them exciting molecules to explore in the field of biomarker discovery.

In particular, due to their small size, they have a high surface area in relation to their volume, resulting in a high surface energy and making them more reactive than the bulk material. Early studies in bio-nano interactions have shown that NPs, after exposure to biological fluid, firmly associate with a layer of biomolecules forming the corona.[1,2] Interestingly, the biomolecular corona composition is strongly related to the NP intrinsic properties as changing the size, surface charge, material, and shape and the corona composition is not correlated with the protein's original abundance in the biological media and the corona composition. Additionally, the corona can also be tuned by changing the ratio of the surface area to protein concentration ratio during the exposure step.[3]

In this study, we show that the biomolecular corona can be tuned to be enriched in a particular set of glycoproteins, such as fibrinogen, to produce a proteomics and glycomics fingerprint that could be used to trace glycoprotein changes. Despite being a more simplified glycan profile compared to full plasma, the corona glycan profile revealed a fibrinogen-derived glycan peak that was found to potentially distinguish lung cancer patients from controls in a pilot study[4].

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(P32) The Library of Brain Glycans

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Glycosylation is one of the most prevalent and diverse forms of post-translational modifications, involving the attachment of complex oligosaccharides - glycans - to protein molecules. Glycans play essential roles in numerous biological processes, including protein folding, cell communication, adhesion, the formation of functional macromolecules, and the regulation of signalling pathways. In addition to contributing to protein stability and function, the diversity of glycosylation structures enables more intricate molecular regulation, which is particularly significant in tissues such as the brain, where precise interactions and signalling are critical for proper function. The brain has a distinctive glycome with many specific structures, including rare and unusual *N*-glycans that are rarely found or completely missing in other tissues.

These organ-specific glycans play a crucial role in the development and functioning of the nervous system, including the proliferation and differentiation of neural stem cells and the guidance of neural pathway development. Although generalist databases and glycan repositories exist, they typically contain only the most commonly occurring structures. Current research in the field of neuroglycobiology is limited by a lack of resources for the precise identification and structural characterization of these brain-specific glycans, which hinders data interpretation and slows progress in studies related to neurological disorders.

To address this challenge, we have developed the Library of Brain Glycans (LBG) - a curated and freely accessible database dedicated to *N*-glycans isolated from brain tissue. The LBG integrates data from various experimental methods, including liquid chromatography and mass spectrometry, and provides information on monosaccharide composition, retention time, molecular mass, and diagnostic fragments for over 350 glycans. In addition to core data, the database also includes metadata such as species, age, brain region and other key biological parameters concerning the expression pattern of individual glycan structures. Visual representations of glycans in the standardized SNFG format allow users to intuitively explore and compare different structures.

One of the key features of the database is its advanced search capability, enabling queries based on physiological variables as well as glycan structure and characteristics. The initial data come from published studies, publicly available resources such as GlycoStore, and our own in-house datasets derived from research involving neurological tissues from various species, including mice, rats, and humans. The library is designed as a "living" platform, to be continuously updated and expanded with new data, with the potential to include other types of glycans in the future. The LBG has a significant potential to accelerate and simplify data analysis in neuroglycobiology.

It enables researchers to identify brain glycans more efficiently, even without access to advanced analytical techniques and serves as a tool for result validation and improved identification accuracy. Our aim was to provide easily accessible resources that support the scientific community, enabling precise data annotation and helping to improve the understanding of brain glycosylation mechanisms. The LBG is freely available at https://lbg.human-glycome.org/.

(P33) Sweet success: The role of sialic acid on T cell function

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Background: Although T cell-based therapies have achieved significant success in certain tumor types, a large proportion of patients receiving cancer immunotherapy still progress with the disease. Thus, there is interest in identifying and blocking alternative pathways mediating immune suppression. Beyond canonical protein-based immune checkpoints such as PD-1/PD-L1 and CTLA-4, tumor-associated glycosylation—particularly hypersialylation—has emerged as a key modulator of immune suppression. Sialic acid-containing glycans on tumor cells are recognized as glyco-immune checkpoints that engage inhibitory Siglec receptors on immune cells. While the immunosuppressive role of tumor sialylation is well established[1], new evidence indicates that sialylation of the T cells themselves may also contribute to immune dysfunction, suggesting a previously underappreciated layer of glyco-regulation in the tumor microenvironment.

Methods: Human T cells were treated with pan-sialidase to enzymatically remove surface sialic acids. Desialylated T cells were then stimulated with anti-CD3/anti-CD28 or co-cultured with bispecific T cell engagers and tumor target cells, followed by flow cytometry. Primary samples from patients with chronic lymphocytic leukaemia (CLL) were analyzed through single-cell RNA sequencing combined with lectin-based glycan profiling.

Results: We discovered that T cells treated with sialidase exhibited enhanced proliferation and activation. In a co-culture system with bispecific T cell engagers and target cells, we demonstrated T cells treated with sialidase showed enhanced cytotoxicity, including increased cytokine production, T cells proliferation, and tumour cell lysis Interestingly, when studying T cell subsets, we observed that sialidase treatment lowered the activation threshold particularly in naïve T cells. Our findings were validated using primary samples from patients with chronic lymphocytic leukaemia (CLL): sialic acid remodelling led to improved immune function.

Conclusion: T cells are extensively decorated with sialic acids, which can modulate their function. Enzymatic removal of these glycans enhances T cell responses and may help overcome resistance mechanisms in cancer immunotherapy. Future studies will investigate whether distinct glycan signatures among T cell subtypes correlate with functional heterogeneity.

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(P34) Chemoenzymatic Glycoengineering of Monoclonal Antibodies: A Novel Solution-Phase Approach for Developing Single Glycoform Therapeutics

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Current approaches for generating single glycoforms monoclonal antibodies (mAbs) include genetic manipulation, cell culture modification, and chemoenzymatic methods. While genetic approaches offer precision when target glycan modifications are predetermined, they are costly and time-intensive. Metabolic approaches provide financial advantages but rarely achieve homogeneity. Chemoenzymatic methods, though expensive, can be retrospectively applied to various therapeutics[1].

Recent chemoenzymatic glycoengineering strategies have utilized glycosyl transferases and hydrolases on solid support membranes or employed endo-β-*N*-acetylglucosaminidase (ENGases) with monosaccharyl transferase mutants[2,3]. Our proposed methodology advances beyond these approaches in two significant ways: 1) implementation of an in solution phase, offering cost advantages and avoiding glycan-site accessibility limitations on intact proteins, often associated with solid support coupling, and 2) utilization of a unique combination of glycosyl transferases and endoglycosidases to generate novel mAb glycoforms.

We will present results on the development of an innovative in-vitro glycoengineering technology enabling solution-phase creation of single glycoforms mAbs with enhanced safety, efficacy, and effector functions compared to heterogeneous counterparts. We will showcase this technology using two different antibodies; an IgG2a mAb developed against the Aspergillus fumigatus siderophore TAFC[4,5] and a polyclonal IgG. We will describe functional assays including FcR binding and kinetic studies with established techniques[6] on the glycoengineered mAbs and comparing results to heterogeneous parent mAbs.

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(P35) Adenosine A2B receptor signalling induces mucin expression in the intestinal epithelium

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Background: Loss of mucosal barrier integrity and inappropriate perpetuation of the inflammatory response are key features of ulcerative colitis (UC). Increased permeability and disruption of the mucus layer lining the colonic intestinal epithelium is observed prior to the onset of colitis in UC and murine colitis models. Therefore, understanding how the integrity of the mucus layer is maintained during health and disease may lead to novel therapeutic approaches for UC.

The major constituents of the mucus layer are mucins which are secreted by goblet cells in the intestinal epithelium. MUC2 is the major mucin within the ileum and colon, and MUC5AC has recently been demonstrated to be protective in murine colitis. Extracellular adenosine signaling can mediate mucin hypersecretion in asthma and other inflammatory respiratory conditions. Studies suggest that signaling via the Adora2b adenosine receptor (A2BAR) is protective in acute gastrointestinal inflammation such as colitis, although the mechanism by which this occurs is not known. We hypothesized that A2BAR signaling induces MUC2 and MUC5AC expression in the intestinal epithelium.

Methods: We are combining human epithelial cell lines and mouse models to investigate the A2B-mediated effects on MUC2 and MUC5AC in the intestine during homeostasis and injury. MUC2 and MUC5AC mRNA expression were quantified by Taqman RT-PCR and MUC5AC protein expression by immunofluorescent staining. To investigate A2B-mediated effects on mucus thickness, growth and penetrability, we use an *ex-vivo* method where the intestinal tissue is mounted in a horizontal perfusion system equipped with temperature control. Mucus thickness, expansion and penetrability to bacteria-sized beads are recorded before and after basolateral and apical stimulation with a selective A2B agonist for a total of 60minutes.

Results: Treatment of human intestinal epithelial cells with an A2B selective receptor agonist induced upregulation of MUC2 and MUC5AC mRNA, which was abolished by receptor blockade and inhibition of PKA. Similarly, stimulation of the A2B-receptor with the selective A2B agonist increased MUC5AC promotor activity. Moreover, we assessed A2B-mediated induction of MUC2 and MUC5AC expression during epithelial injury. A2B agonist treatment of wounded intestinal epithelium resulted in increased MUC2 and MUC5AC mRNA expression and MUC5AC protein expression. We further explored the A2B-mediated effects on mucus growth and penetrability during homeostasis by an *ex-vivo* approach.

Conclusions: Our preliminary findings suggest that signaling through the A2BAR can increase MUC2 and MUC5AC expression in the intestine during homeostasis and injury.

(P36) Optimization of Analytical Techniques for Mucin Investigation

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Mucins are heavily glycosylated proteins that play crucial roles in biological processes such as lubrication, cell signalling, and pathogen defence[1]. Given their complex structure and glycan heterogeneity, optimizing analytical techniques for mucins is essential for advancing biomedical research and biotechnology applications. This study aims to refine gel electrophoresis, staining procedures, and monosaccharide analysis for mucins derived from human saliva, porcine gastric mucin (PGM), and jellyfish extracellular matrix (ECM). By standardizing these methods, we seek to enhance the resolution, sensitivity, and reproducibility of mucin characterization across different biological sources.

Gel electrophoresis remains a fundamental tool for analysing mucins, yet conventional protocols often yield poor resolution due to their high molecular weight and glycosylation complexity. In this project, we explore variations in gel polymers, acrylamide concentration, buffer compositions, and electrophoretic conditions to optimize separation efficiency.

Staining mucins effectively poses another challenge, as traditional protein stains such as Coomassie Brilliant Blue exhibit low affinity for glycosylated proteins. To address this, we compare periodic acid-Schiff (PAS) staining and Alcian Blue to identify the most effective method for detecting mucins with high sensitivity and specificity.

Monosaccharide composition analysis provides critical insights into mucin glycosylation patterns, which influence their biological function. We optimize acid hydrolysis conditions, derivatization techniques, and chromatographic separation methods for precise quantification of monosaccharides from mucin samples.

A key aspect of this project is the comparative analysis of mucins from saliva, PGM, and jellyfish ECM. Human salivary mucins are known for their protective roles in the oral cavity, while PGM is widely used as a model mucin in biomedical research. Jellyfish ECM represents a unique and underexplored source of mucins, potentially offering novel structural and functional properties. By systematically evaluating these mucins under optimized experimental conditions, we aim to identify commonalities and differences that could inform their functional applications in biomedicine, bioengineering, and material science.

Through this study, we establish a robust framework for mucin characterization, facilitating more accurate and reproducible analyses across diverse biological sources. The findings will support advancements in mucin-based therapeutics, biomaterials, and diagnostic tools. Future work will focus on integrating glycoproteomic approaches and structural analysis techniques to further elucidate mucin architecture and function. This project ultimately aims to refine mucin analysis methodologies, contributing to a deeper understanding of their role in health and disease.

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(P37) Glycoproteins at the host/parasite interface: Study of the filarial nematode Brugia malayi excretome/secretome

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Filarial nematodes are widely spread infectious organisms that represent a major human, social and economic burden in developing countries. It is well established that these parasitic worms survive for extensive periods of time (often over a decade) in their mammalian host using various mechanisms of immune evasion. Excretory/secretory (ES) products of diverse nature, including proteins, play major parts in these processes.

Previous studies of *Brugia malayi* have shed light on ES proteins of this causative agent of human lymphatic filariasis, revealing life-stage and sex specificities[1-3]. While a large proportion of the ES proteins are expected to be glycosylated, characterisation of the glycan moieties of the *B. malayi* ES products is currently lacking. Glycans, however, are known to confer pivotal functional properties to the proteins they modify, and filarial nematode glycans encompass phosphorylcholine (PC)-substituted structures, known to be necessary for the induction of characteristic T helper 2 immune responses.

Here, we applied mass spectrometry (MS)-based glycomic techniques to define the *N*-linked and *O*-linked glycans decorating the ES products of *B. malayi* adult females and microfilariae. ES proteins were found to be preferentially modified by glycans containing zwitterionic and anionic motifs with known immunogenic and immunomodulatory properties. Notably, previously undetected multiantennary *N*-glycans containing terminal glucuronic acid and PC substituents were defined using glycan sequencing combined with tandem MS. The high abundance of these complex, acidic structures in the ES compared to the total worm *N*-glycome indicates a role for this subset of glycans in host-parasite interactions. In addition, we precisely mapped the characterized *B. malayi* ES glycome into its proteome using glycopeptide enrichment techniques combined with up-dated MS workflows.

This work uncovers the striking heterogeneity of protein glycosylation and provides precise characterisation of the glycosylation of individual proteins including known and potential immunomodulators. Altogether, this comprehensive glycoproteomic dataset consolidates our knowledge of *B. malayi* ES products and provides information relevant to the identification of vaccine and diagnostic targets.

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(P38) Streamlining Complement component 3 N-glycan analysis to enhance diagnostic potential

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Complement component 3 (C3) is a key molecule of the innate immune system, involved in activation of the complement system via both classical and alternative pathways. Human isoform of C3 protein has three consensus N-glycosylation sites, two of which are known to be glycosylated: Asn63 and Asn917.

Our research group recently developed a high throughput method for the analysis of C3 *N*-glycosylation and used it to investigate its changes in type 1 diabetes[1]. We've revealed changes in children with type 1 diabetes compared to controls as well as in an adult population[2], demonstrating both a significant prognostic and diagnostic potential of C3 N-glycans. The method relied on enrichment of C3 from human plasma using lectin affinity followed by bottom-up glycoproteomics using Glu-C digestion. The analysis was done on a UPLC-ESI-qTOF-MS.

This method, although offering reasonably high-throughput and repeatability, still poses challenges when considering its possible application in a wider clinical setting. The cost of analysis, both in terms of labour and material could be significantly reduced making it more appealing for use in a clinical laboratory. Besides the qTOF MS instruments, many laboratories have a triple quadrupole (QQQ) instrument which offers superior specificity and quantitative potential at the cost of mass accuracy and level of information provided. In the research we are presenting here, we've successfully transferred the UPLC-ESI-qTOF method for C3 *N*-glycopeptide analysis to a UPLC-ESI-QQQ instrument. We've optimized the voltages for the collision and fragmentor as well as ascertained optimal mass transitions for MRM analysis. Three N-glycans on each glycosylation site were successfully detected. Structure of these glycopeptides was first confirmed by doing a product ion scan, using which optimal fragment ions were selected for each parent ion. Optimal mass transitions are as follows: Asn63.N2H5 933.5→906, Asn63-N2H6 974→906, Asn63-N2H7 1014.5→906, Asn.917-N2H8 877.5→1060.9, Asn917-N2H9 918→1115, Asn917-N2H10 958.6→1115. These fragments originate form glycan B or Y fragments with the peptide backbone remaining intact at the selected voltages.

We have also streamlined the sample preparation for the qTOF method by removing the lectin enrichment of C3 and analysing glycopeptides by direct digestion of plasma proteins with Glu-C. This significantly reduces sample preparation time from 3 days to 2 days, and hands-on time in the lab by more than half. Material cost was reduced by approximately one third, primarily by eliminating the need for Concanavalin A Sepharose beads for lectin affinity enrichment. Six C3 glycopeptides (three on each site) were qualitatively confirmed using measured mass, retention time and MS/MS fragmentation spectra.

Quantitative evaluation and comparison of the two methods as well as the original method will be carried out in the future. Adaptations described herein greatly increase the clinical potential of C3 N-glycopeptide analysis.

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(P39) Comparison of *E. coli* BL21(DE3) and *E. coli* Rosetta-Gami B for the Expression of PNGaseF Using the Plasmid OmpA3-PNGaseF-TEV-His6

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Protein expression in *Escherichia coli* (E. coli) remains a widely used approach for recombinant protein production due to its rapid growth, ease of genetic manipulation, and cost-effectiveness[1]. However, differences in *E. coli* strains can significantly impact the yield and solubility of heterologous proteins. In this study, we compare the expression of Peptide-N-Glycosidase F (PNGaseF), an important enzyme used for glycoprotein deglycosylation, in two commonly used *E. coli* strains: BL21(DE3) and Rosetta-Gami B. Both strains were transformed with the plasmid OmpA3-PNGaseF-TEV-His6[2], which contains an OmpA3 signal sequence for periplasmic targeting, a TEV protease cleavage site for tag removal, and a His6 tag for affinity purification.

BL21(DE3) is a widely used strain for recombinant protein expression, characterized by its deficiency in Lon and OmpT proteases, which helps to reduce protein degradation, and its chromosomally integrated T7 RNA polymerase gene, allowing for strong induction of protein expression. However, it lacks tRNAs for rare codons and does not enhance disulfide bond formation, potentially limiting efficient translation and proper folding of certain heterologous proteins. In contrast, Rosetta-Gami B is a modified strain that combines the benefits of Rosetta (which carries a supplementary plasmid encoding tRNAs for rare codons) and Gami B (which enhances disulfide bond formation by mutating thioredoxin reductase and glutathione reductase genes). These modifications make Rosetta-Gami B an attractive choice for proteins requiring improved folding conditions and rare codon adaptation.

Expression levels of PNGaseF were assessed in both strains under induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). SDS-PAGE was used to quantify total protein yield and solubility.

Initial results indicate that BL21(DE3) strain is a better choice for expression of PNGaseF, however optimisation studies are ongoing. Our results will highlight the importance of strain selection in optimizing recombinant protein production, particularly for proteins requiring rare codon adaptation and proper disulfide bond formation. Future work may explore additional optimization strategies, including co-expression with chaperones or alternative signal sequences, to further improve periplasmic targeting and protein folding.

Overall, this study provides valuable insights into strain-dependent factors influencing PNGaseF expression and underscores the benefits of utilizing modified B *E. coli* strains for proteins with rare codon enrichment and complex folding requirements. These findings may have broader implications for recombinant protein production, particularly in biopharmaceutical and structural biology applications where efficient protein expression is critical.

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(P40) Stem Cell-Derived Intestinal Organoids for Evaluating Oestrogen's Protective Effects and Glycopattern Modulation in Intestinal Inflammation

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Oestrogen is a key regulator of intestinal permeability, acting through oestrogen receptors α and β, which are expressed in both epithelial and immune cells of the intestinal environment[1]. Variations in oestrogen levels throughout life stages, such as puberty, the menstrual cycle, pregnancy, and menopause, may influence intestinal barrier function and immune responses, contributing to sex-related differences in inflammatory bowel conditions[2]. Although preclinical animal studies have shown that oestrogen supports epithelial integrity and modulates immune signalling[3], these models lack a physiologically relevant human endocrine system, limiting their translational potential. The precise mechanisms underlying oestrogen influence on the intestinal barrier remain poorly understood.

To address this gap, human intestinal organoids (HIOs) derived from induced pluripotent stem cells (iPSCs) from female donors were employed. These organoids were exposed to inflammatory stimuli (e.g., IFN-γ) and oestrogen to investigate their combined effects on barrier function and immune regulation. Oestrogen receptor β expression alongside key tight junction markers, such as E-cadherin and ZO-1 were evaluated. Moreover, given the critical role of glycosylation in maintaining the mucus barrier and epithelial glycocalyx and considering the impact of inflammatory processes in modulating the intestinal glyco-environment, characterized by the exposure of Galβ1-3GalNAc-O-Ser/Thr terminal residues[4], lectin staining was performed to analyse glycopatterns and assess glycan expression changes in response to oestrogen and inflammatory conditions.

This approach provided novel insights into oestrogen role in modulating glycan structures that contribute to mucosal protection. Finally, inflammatory cytokine profiles were further examined, and co-culture experiments with macrophages were conducted to explore the effects of oestrogen on immune responses. Transcriptomic and proteomic analyses were carried out to dissect oestrogen-mediated signalling pathways.

These findings offer a deeper understanding of the interplay between oestrogen, inflammation, and glycosylation, with potential implications for developing sex-specific treatments for intestinal inflammatory disorders.

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(P41) Patient-derived organoids as an *ex vivo* model to study gastric glycosylation dynamics

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Aberrant glycosylation plays an active role in gastric carcinogenesis[1]. Patient-derived organoids (PDOs) have emerged as powerful models in pre-clinical and translational research to study human gastric disorders. These 3D models retain key genetic and phenotypic characteristics of their tissue of origin. However, their glycosylation landscape remains unexplored. Therefore, we aim to validate PDOs as representative models of the glycosylation profile of *in vivo* tissues.

A gastric PDOs biobank (n=56) was established, comprising PDOs derived from fresh gastric mucosa of non-tumoral patients (n=11), from adjacent tumour mucosa (n=26), and tumour tissue (n=19) of gastric cancer (GC) patients. Additionally, our biobank also includes paired adjacent normal and *sibling* tumour PDOs derived from the same patients. The glycosylation profile of PDOs and their respective parental tissues were thoroughly characterized, followed by a comparative glycan analysis over time, upon PDO biobanking and xenografting in mice. The binding of *two Helicobacter pylori* (*H. pylori*) isogenic strains with distinct glycan-binding affinities was assessed on parental tissue and upon modulation of the gastric mucosa PDO glycan landscape.

Our results demonstrate that PDOs are *avatars* of the *in vivo* glycosylation profile, mimicking the glycan phenotype observed in the gastric carcinogenic cascade. Importantly, this feature was maintained over time, upon biobanking and xenografting. A comparative analysis of sibling PDOs and their corresponding adjacent PDOs showed the inter- and intra-tumour glycan heterogeneity characteristic of GC. Additionally, we demonstrate that Lewis type I and Lewis type II antigens can be modulated according to PDOs differentiation status, aligning with *H. pylori's* binding to specific Lewis antigens, mirroring *in vivo* tissue interactions.

These findings demonstrated that PDOs are a valuable tool to study the complex glycan dynamics in both gastric physiological and pathological settings[2], opening new opportunities to develop glycan-based therapeutic approaches[3].

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(P42) Glycan sequencing with ion mobility-mass spectrometry

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Glycans play essential roles in biological processes, and understanding their biology at a molecular level is key for developing biopharmaceuticals, diagnostics and nutraceuticals. It is therefore essential to accurately determine exact glycan structures in complex biological samples. While mass spectrometry (MS) offers compositional and structural insights, it struggles with isomeric glycans. Here, we introduce an ion mobility (IM)-MS method for rapid glycan identification and *de novo* sequencing, accurately distinguishing isomeric structures across glycan classes using a limited set of synthetic standards.

We chemoenzymatically synthesized a collection of glycan standards encompassing all glycan classes and used these to develop the IM-MS method. IM uses a drift cell filled with an inert gas to separate ions within an electric field based on their shape, enabling isomer separation. Ion activation prior to IM separation yielded unique arrival time distributions (ATDs) and collision cross section (CCS) values for intact glycans and fragments, contributing to a self-expanding glycan reference database. The methodology was used to identify *N*- and *O*-glycans on glycoproteins, glycan moieties on glycosphingolipids and free human milk oligosaccharides (HMOs) in milk.

Glycans form stable gas-phase conformations during electrospray ionization, producing diagnostic IM-ATD fingerprints. An ATD database of synthetic standards enabled rapid structural characterization in biological samples[1]. For unknown glycans without an entry in the database, a *de novo* sequencing method was developed. We synthesized a library of standards with common glycan epitopes from different glycan classes and subjected them to ion activation, yielding a database with CCS values of glycan subunits. This allowed for e.g. the identification of isomeric *O*-acetyl positions on sialic acids from *O*- and *N*-glycans in different biological samples[2]. By identifying additional subunits, we *de novo* sequenced their full structures and incorporated their CCS values, along with those of the other subunits, into the database. This approach gradually facilitates the sequencing of more complex structures. In this way, we identified 19 *N*-glycans and 43 HMOs from biological sources for which no synthetic standards were available. For *O*-glycan analysis, we developed an oxidative release method that yielded a carboxylic acid moiety on the reducing end[3]. We synthesized all eight *O*-glycan cores with the same moiety, which allowed us to identify these and extended structures in biological samples with IM-MS. For glycosphingolipids, we added two isomeric ganglio-series glycans to the database. With subunits from these isomers we elucidated all major ganglio-series glycans and analyzed glycosphingolipid expression in neuroblastoma organoids. This IM-MS methodology is expected to empower the glyco-research field with rapid and precise identification of glycan structures in biological samples, eliminating the need for large collections of synthetic standards.

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(P43) N-Glycome Characterisation of the Human Cornea

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Introduction: The human cornea is a transparent, avascular tissue at the anterior segment of the eye, facilitating the passage of light. Neovascularisation, the ingress of blood vessels into the corneal stroma, disrupts 'immune privilege', and is a risk factor for the rejection of corneal transplants[1]. There is a pressing need for novel markers and therapies for corneal neovascularisation. N-glycosylation, the post-translational modification of the asparagine (N) residues of proteins with complex carbohydrates called glycans, modulates the structure and function of proteins. Changes in tissue N-glycosylation are associated with pathology and can serve as 'glycosignatures' of disease, acting as targets for novel 'glycotherapies'. For example, increases in glycan branching (tri- & tetra-antennarity) and fucosylation have been reported in a range of pathologies[2, 3]. Understanding how these glycan traits change in disease allows for the development of novel diagnostics and treatments[4,5,6]. The role of glycan-binding galectin proteins in corneal neovascularisation is well characterised[7]. However, the underlying corneal N-glycome and how it changes in neovascularisation is not as well understood. To address this gap, we performed a comprehensive analysis of the *N*-glycome in healthy and neovascularised human corneal samples.

Objectives & Methods: The objective of this study was to carry out a first-of-its-kind characterisation of the human corneal N-glycome, and to determine how it is altered in pathological neovascularisation. To identify these glycosignatures, healthy human corneas from deceased donors, and neovascularised corneas from transplant patients were collected. Snap-frozen tissues were used for whole N-glycome profiling via HILIC-UPLC and LC-MS[8]. Formalin-fixed tissues were then used for MALDI spatial imaging of N-glycans in the cornea[9]. Human corneal sections were also analysed by lectin histochemistry and confocal microscopy[10]. Cytokine-stimulated primary human corneal fibroblasts (HCFs) were treated with a fucosylation inhibitor to analyse potential glycotherapies for corneal disease, along with a TGF-β1 model of fibrosis and *in vitro* angiogenesis with HUVECs[11].

Results: HILIC-UPLC and LC-MS analysis of the human corneal *N*-glycome revealed a diverse array of >120 *N*-glycans. Fucosylated, sialylated and branched *N*-glycans were significantly upregulated in neovascularisation. Spatial MALDI imaging demonstrated that the corneal stroma was the primary site of *N*-glycosylation, with the distribution of these *N*-glycans being dysregulated in neovascularisation. Lectin histochemistry of human corneal tissue sections also demonstrated an upregulation of glycosylation in the neovascularised stroma, particularly fucosylation. Cytokine-induced inflammation of HCFs also caused an upregulation of fucosylation, as indicated by lectin histochemistry. When HCFs were treated with a metabolic inhibitor of fucosylation, fibrosis and metabolic dysfunction were reduced, as shown by fibrotic markers, migration and Seahorse metabolic flux assays. Angiogenesis was also reduced, as indicated by HUVEC tube formation and 3D sprouting.

Conclusions & Significance: These data represent a first-of-its-kind characterisation of the human corneal N-glycome and show that changes in *N*-glycosylation (fucosylation and antennarity) constitute glycosignatures of corneal neovascularisation, and that the inhibition of fucosylation can serve as a glycotherapy for this pathology.

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(P44) Experimentally guided reconstruction of glycoproteins 3D structures with GlycoShape ReGlyco-Fit

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Despite major advancements in structural biology and computational modelling, resolving glycan structures and their occupancy from solution scattering remains a long-standing challenge. The inherent flexibility of glycans and the low-resolution nature of small-angle X-ray scattering (SAXS) have historically limited its use to detecting glycan presence rather than extracting detailed structural information.

Here, we introduce Re-Glyco Fit, a computational framework that leverages GlycoShape, an extensive molecular dynamics (MD)-generated glycan conformation database, to bridge the structural gap in SAXS data interpretation. By systematically fitting experimental SAXS profiles to a library of precomputed glycan structures, we infer glycan size categories (small, medium, large) and site occupancy, offering a practical approach to characterizing glycoproteins in solution. Our method shifts SAXS from a validation tool to an inference-driven strategy, demonstrating that the integration of glycan flexibility constraints significantly improves structural predictions. We validate our approach across diverse glycoproteins, showing alignment with known glycosylation patterns and providing a rapid, cost-effective alternative to labour-intensive gold-standard techniques such as LC-MS glycoproteomics.

This work establishes a new paradigm for SAXS-based glycan modelling and lays the foundation for future methods that incorporate glycan structural diversity in cryo-EM and other low-resolution structural techniques.

(P45) Population-scale analysis of complement C3 *N*-glycosylation reveals immune pathway regulation and disease associations

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While glycosylation's role in antibody effector functions is well-characterized, its significance for complement component C3—the central mediator of all complement activation pathways essential for pathogen defence and immune homeostasis—remains largely unexplored[1]. We performed high-throughput LC-MS analysis to characterize *N*-glycosylation at two C3 glycosylation sites (N63 and N917) in 816 adults, combined with genome-wide association analysis to identify genetic regulators.

Our analysis revealed a unique glycosylation profile exclusive to C3, characterized by nine high-mannose glycoforms with complete site occupancy. Unlike other plasma glycoproteins, C3 retains monoglucosylated glycans typically associated with ER quality control, suggesting specialized regulatory mechanisms. Molecular dynamics simulations demonstrated that C3's protein structure restricts glycan processing enzyme accessibility, explaining the persistence of these high-mannose structures. Structural modelling of the iC3b-CR3 immune receptor complex revealed direct interactions between the N63 glycan on C3 and potential N224 glycan on CR3[2], suggesting that glycan-glycan contacts could modulate immune cell recognition. These glycans could form close contacts regardless of glycoforms type, with their positioning restricted by the protein interface, providing a mechanistic link between glycosylation and immune function. Sex-specific differences in C3 glycosylation were particularly pronounced, with males showing higher prevalence of less processed glycan structures compared to females. These patterns may contribute to sex-specific differences in complement activation.

Genome-wide association identified six genetic loci regulating C3 glycosylation, encompassing genes involved in glycan processing, protein secretory pathways, and complement regulation. Notably, we discovered strong genetic colocalization between C3 *N*-glycosylation and both rheumatoid arthritis (PP4=91%, probability of shared causal variant) and inflammatory bowel disease (PP4=65%), indicating shared causal variants and suggesting C3 glycosylation may represent a previously unrecognized disease mechanism.

Our findings establish C3 *N*-glycosylation as a potential regulatory mechanism in complement pathway function and a promising therapeutic target in immune diseases.

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(P46) Targeting the 'sweet spot' of metastatic breast cancer: a chemical glycobiology approach

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Protein glycosylation is one of the most abundant and complex post-translational modifications, mediated by the interplay of over 250 glycosyltransferase enzymes. Glycans are displayed on the surface of every single cell, where they modulate a range of cell-cell and cell-matrix interactions. A common subtype is *O*-GalNAc glycosylation. This involves the formation of an *O*-glycosidic bond between the monosaccharide N-acetylgalactosamine (GalNAc) and the hydroxyl group of a serine or threonine residue on a protein backbone. This process is catalysed by a family of enzymes called GalNAc- transferases (GalNAc-Ts). The GalNAc-T family consists of 20 members encoded by the human genome

Aberrant *O*-GalNAc glycosylation is seen in over 90% of breast cancers. Although it has been demonstrated that changes in *O*-GalNAc glycosylation promotes cancer progression and malignancy, the molecular details of the glycoproteome alterations that take place during early disease and metastatic progression remain unknown. It is known that several GalNAc-Ts are overexpressed in breast cancer. However, challenges in studying the substrate specificity of GalNAc-Ts prevail, due to overlapping glycosylation sites and substrate redundancies. Furthermore, no consensus motif for addition of GalNAc to polypeptides currently exists, making our overall understanding of the functional role of each enzyme difficult.

To investigate the role of each GalNAc-T, we equipped breast cancer cell lines with the ability to tag glycoproteins with a traceable, bio-orthogonal chemical group. We achieved this by introducing an artificial biosynthetic pathway to cells, consisting of engineered glycosyltransferases and chemically modified monosaccharide analogues which can then be transferred to their protein substrates. We then developed reagents which allow derivatisation of the chemically tagged glycoproteins and subsequent characterisation by MS-glycoproteomics. These strategies enabled us to uncover promising target proteins in breast cancer. Furthermore, we demonstrate that this approach is amenable to in vitro co-cultures, ex vivo and in vivo studies, thus allowing us to design clinically relevant models that recapitulate a tumour microenvironment.

We are using these methods to understand the link between glycosylation and breast cancer. Profiling the glycoproteome to uncover target proteins in disease progression may help yield new diagnostic or therapeutic options towards breast cancer.

(P47) Using polysialylated bioconjugates for immunological applications

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The carbohydrate polymer polysialic acid (polySia) interacts with a variety of immunologically relevant molecules leading to a modulation of the immune response. One example is the interaction with histones in neutrophil extracellular traps (NETs). The release of NET is an important mechanism of neutrophils to prevent a rapid pathogen invasion. It consists of DNA and several antimicrobial molecules, such as histones, to catch and kill pathogens. However, NETs can also be harmful for the host, since the DNA meshwork can support the formation of microthrombi in small blood vessels. Moreover, extracellular histones show cytotoxic effects against body's own cells, which can enhance the inflammation. Remarkably, the binding of polySia decreases the cytotoxic capacity of histones against eukaryotic cells, while not affecting their antimicrobial effects.

Because of these advantageous properties of polySia, we developed different polysialylated bioconjugates to target histones in NETs. For instance, polysialylated streptavidin was applied in combination with biotinylated fluorescein isothiocyanate for the visualization of NET fibers. Moreover, we investigated novel strategies to degrade NET using polysialylated DNase. The polySia chains on the DNase have the following two functions: mediating an effective accumulation of DNase in NET and inactivation of the cytotoxic histones. Thus, our aim is to use polysialylated DNase to prevent the formation of microthrombi by the simultaneous inactivation and degradation of NET.

(P48) *O*-glycan sequencing with ion mobility mass spectrometry

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The structural diversity of *O*-glycans, combined with the lack of specific enzymes for their release, presents a significant challenge for their unambiguous characterization. We recently developed a neutral oxidative release method for *O*-glycans that preserves serine- and threonine-derived information by forming glycolic and lactic acid moieties at the reducing end[1] while also preventing the peeling reactions commonly observed with alkaline release methods. For the identification of sialic acid linkages and acetylation patterns we developed an ion mobility mass spectrometry (IM-MS) method to obtain intrinsic collision cross-section (CCS) values for identification[2]. Here, we present a modified release method, combined with IM-MS, to accurately identify full *O*-glycan structures with IM-MS, using a library of chemoenzymatically synthesized *O*-glycan standards.

We improved the release method and applied a labelling approach for released glycans for enhanced positive ion mode fragmentation, IM-MS and fluorescence detection, enabling accurate *O*-glycan identification and quantitative analysis. The method was developed using a chemoenzymatically synthesized library of *O*-glycans with core 1–8 structures and extended motifs, with glycolic and lactic acid residues at the reducing end.

We combined these standards with collision cross-section measurements in IM-MS to achieve high-resolution structural determinations of both intact glycan ions and their fragment ions. In parallel, our group has previously established terminal epitope libraries for classes such as *N*-glycans and human milk oligosaccharides. By adding the newly released *O*-glycan core structures, we fill crucial gaps in our self-expanding reference library, which accelerates the identification of unknown structures through matching collision cross-section values and fragmentation patterns. Through application, we identified multiple isomeric *O*-glycan core structures in diverse mucin-rich samples, for example, Core 5 was found in relatively high abundance in pulmonary mucus. Ongoing adaptations to the method further enable semiquantitative analyses for improved comparisons across different mucus types.

Applying this method to mucin-rich samples from various anatomical sites and species enables the direct confirmation of *O*-glycan structures, eliminating the need for solely biosynthetic reasoning. This approach will facilitate the step from compositional analysis to true structural determination of *O*-glycans.

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(P49) A genetically engineered cell-based platform to study interplay of tyrosine sulfation and protein *O*-glycosylation on chemokine receptors

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The interaction between chemokines and their receptors is responsible for several biological functions occurring during an inflammatory response, such as the directed movement of leukocytes to injury sites[1]. Increasing evidence highlights the presence of potential tyrosine sulfation and *O*-glycosylation sites in the N-terminal domain of chemokine receptors, which are described to enhance the binding affinity to chemokine ligands[2,3]. However, the biological role of these two posttranslational modifications (PTMs) and the interplay between each other is still unknown in this context.

The main goal of this PhD project is to produce and analyse a novel cell display platform to study the impact of tyrosine sulfation and *O*-glycosylation on chemokine receptors and dissect their roles in chemokine functions.

To achieve this, we are performing combinatorial knock-out and knock-in of tyrosylprotein sulfotransferases (TPST1 and TPST2) and create new connections with the previously established *O*-glycosylation cell-display library[4]. This cell-based platform is then being used to display and produce protein reporters containing the N-terminal domain of players in the immune system, namely P-selectin glycoprotein ligand-1 (PSGL-1) and C-C chemokine receptor type 5 (CCR5).

Thus, mass spectrometry techniques will be used to characterize the glycosylation and sulfation patterns of the reporters, allowing us to genetically dissect the role of the enzymes involved in the two PTMs in study as well as studying the cross-talk between each other. Moreover, our cell-display platform will be used in several functional assays, including the assessment of the impact of tyrosine sulfation and *O*-glycosylation to the binding to chemokine ligands by flow cytometry and surface plasmon resonance.

Overall, this project, under the scope of the B-ACTIVE MSCA Doctoral Network, will enable a better understanding of the mechanisms that regulate the activity of chemokine receptors, with the potential of finding novel drug targets to fight uncontrolled and excessive inflammatory responses.

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(P50) Sialylation is differentially expressed in M1-like and M2-like human monocyte-derived macrophages

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The phenotype of a cell is largely restricted by its corresponding protein expression. Post-translational modifications play a critical role in protein folding, trafficking, activity, and function[1,2,3]. Glycosylation, the covalent attachment of sugar residues to the protein backbone, is one of the most abundant forms of post-translational modification (PTM). Cell surface glycans regulate fundamental biological processes, such as adhesion, survival, migration, and host protection against pathogens[4-13] However, the impact of cell surface glycosylation on material-cell interactions remains largely unexplored.

Hence, we hypothesized that inherent, sustainable, glycan-driven bioactive cues can modulate the biomaterial-host response. Using human monocyte-derived macrophages, we identified distinct glycosylation signatures associated with proinflammatory (M1-like) and anti-inflammatory (M2-like) phenotypes. M2-like macrophages showed an upregulation of α,2-6 sialylation (SNA) and its associated enzyme ST6GAL1, while M1 macrophages exhibited an upregulation of α,2-3 sialylation and its associated enzyme ST3GAL1 respectively. Additionally, both phenotypes exhibited downregulation of α,2-3 sialylation (MAL I) and mannosylation (GNL).

Furthermore, global transcriptomic profiling of Glycan-Binding Proteins (GBPs) showed Siglec-1 upregulation in M1-like macrophages and Siglec-5 downregulation in M1-like macrophages.

This research elucidates the potential of glycosylation to modulate immune-biomaterial interactions, thereby presenting opportunities for the development of glyco-modified biomaterials designed to attenuate adverse immune responses commonly associated with implanted devices. This approach offers a promising strategy to mitigate fibrotic encapsulation and direct immune responses towards pro-regenerative outcomes in biomaterial-based therapies.

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(P51) IgG Fc glycosylation and subclass distribution differ between anti-centromere protein B and anti-topoisomerase I autoantibodies in systemic sclerosis

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Systemic sclerosis (SSc) is a complex autoimmune disorder characterized by vasculopathy, immune dysregulation, and fibrosis of the skin and internal organs.[1] Clinical presentation of SSc vary from an indolent form with limited deterioration to rapidly progressive disease, associated with irreversible organ damage and relatively high mortality.[2] This heterogeneity poses significant clinical challenges with currently very limited effective treatments to prevent disease progression.[3] Autoreactive B cells are considered to play a major role as B cell depleting therapies improve clinical manifestations.[4] Furthermore, over 95% of SSc patients harbor antinuclear antibodies, including antibodies targeting centromere protein B (ACA) and topoisomerase I (ATA). These two disease-specific autoantibodies rarely co-occur and are associated with distinct clinical phenotypes, suggesting differences between the underlying mechanisms driving ACA^+ and ATA^+ SSc.[5]

Although ACA and ATA have been extensively used as diagnostic and prognostic markers, limited is known about their characteristics and how these antibody features may impact clinical outcome. In rheumatoid arthritis (RA), it is well established that anti-citrullinated protein antibodies (ACPA) exhibit distinct Fc glycosylation patterns—characterized by decreased galactosylation and increased fucosylation—compared to total IgG from both healthy donors and RA patients.[6] These G0F glycosylation profiles are associated with inflammation and disease progression. Additionally, IgG G0F Fc glycans may modulate IgG effector functions due to fucose impairing Fcγ receptor III binding and lack of galactose, resulting in diminished hexamerization, C1q binding, and complement activation.[7] However, it remains unclear if ACA and ATA in SSc exhibit similar alterations in Fc glycosylation.

To this end, we characterized the Fc glycosylation profiles and subclass usage in total IgG and antigen-specific IgG in a cohort of ACA^+ (n = 37) and ATA^+ (n = 32) SSc patients. These patients, with a disease duration of less than three years and minimal immunosuppressive treatment, were compared to age- and sex-matched healthy donors (HD, n = 30). After isolating both total and antigen-specific IgG followed by trypsin digestion, the resulting glycopeptides were measured using liquid chromatography–mass spectrometry (LC-MS). Across most IgG subclasses, total IgG Fc glycans from SSc patients are less galactosylated and sialylated, and exhibit more bisecting GlcNac compared to healthy donors. Moreover, comparison of antigen-specific IgG1 to total IgG1 within the same patient, both ACA- and ATA IgG1 were found to be more fucosylated, bisected, agalactosylated, and asialylated than total IgG1. Interestingly, a direct comparison between the two SSc subtypes revealed that ACA IgG1 exhibited significantly more bisecting GlcNac and sialylation per galactose residue than ATA IgG1. In addition, while respectively 69% and 41% of ATA^+ patients showed detectable ATA IgG2/3 and IgG4, only 8% and 5% of ACA^+ patients had detectable IgG2/3 and IgG4, with ACA IgG predominantly confined to the IgG1 subclass.

To validate the latter findings, we performed total and antigen-specific IgG ELISAs across both pan IgG and IgG subclasses. Total IgG concentrations were higher in ATA^+ patients (median: 20.2 mg/mL) compared to ACA^+ patients (median: 13.8 mg/mL) and healthy donors (median: 12.9 mg/mL). No correlation was found between total IgG and antigen-specific IgG levels as measured with ACA- and ATA-ELISA. Consistent with our LC–MS findings, ATA^+ samples exhibited a broader IgG subclass distribution. Antigen-specific IgG2 and IgG3 levels were detected 59% and 53% of ATA^+ patients, respectively, whereas ACA IgG2 and IgG3 were detected in only 19% and 11% ACA^+ patients. In contrast, ACA IgG was predominantly confined to the IgG1 subclass, with a minor presence of IgG4,

highlighting a more restricted subclass profile in ACA^+ patients compared to the broader profile observed in ATA^+ individuals.

In conclusion, our study shows remarkable differences in the autoantibody response between ACA^+ and ATA^+ SSc. Specifically, ACA IgG1 exhibits more bisecting GlcNac and more sialylation per galactose than ATA IgG1. Moreover, ACA IgG is predominantly found as IgG1, in contrast to the broader subclass distribution observed for ATA IgG. These findings suggest that the ACA and ATA responses are differentially regulated, which may contribute to understanding the distinct disease courses observed in ACA^+ versus ATA^+ patients.

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(P52) Alterations in *N*-glycome composition during human fetal development

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The development of the human fetal immune system, particularly in the first and second trimesters remains poorly understood. During pregnancy, in-utero exposure to various drugs and chemicals through maternal smoking, alcohol consumption, drug abuse, prescription medications, and environmental pollutants is widespread. These exposures are linked to major health conditions, and can impact fetal immune development and metabolic programming, with potential lifelong consequences. Understanding normal developmental processes and the impact of prenatal exposures on the fetal immune system and metabolism is therefore essential. In this context, the dynamics of protein glycosylation alterations during fetal development, and its response to various exposures, has not been sufficiently investigated. Glycome, the complete set of glycans attached to proteins, is shaped by genetic, epigenetic, and environmental factors. Thus, altered glycosylation patterns may indicate pathological changes and could serve as both biomarkers and functional regulators of physiological processes.

In this collaborative research, we analysed *N*-glycome composition of human fetal liver and placenta samples (142 in total) from both male and female fetuses at 7-20 weeks of gestation. These samples were collected from normally progressing, electively terminated pregnancies as part of the Scottish Advanced Fetal Research Project (SAFeR) study, providing a unique opportunity to investigate the relationships between glycosylation, fetal age, sex, and maternal lifestyle factors. Specifically, proteins were extracted from 36 female and 35 male placenta and liver samples and their glycosylation was investigated using hydrophilic interaction ultra-high-performance liquid chromatography (HILIC-UPLC) analysis.

Our analysis revealed extensive significant associations between gestational age and tested glycosylation patterns. Specifically, 21 out of 45 tested glycan features originating from liver proteins and 40 out of 72 glycan features originating from placenta proteins exhibited a significant correlation with gestational age. In contrast, the effects of fetal sex and maternal smoking were only nominally significant. The observed striking glycosylation changes could potentially reflect the fact that many new glycoproteins emerge as the tissue development progresses. Given the essential role of glycans in almost all biological processes, this area of research opens up new avenues to explore mechanisms of fetal programming effecting long-term health outcomes, and warrants further investigation.

(P53) TnMiner: Technology for the discovery of tumour specific glycopeptide epitopes

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The field of anti-tumour immunotherapeutics is expanding very rapidly, with the development of many new potent modalities over the past decade, such as antibody-drug conjugates (ADC), chimeric antigen receptor therapy (CAR), bispecific T-cell engagers (BiTes) and many derivatives thereof. However, the discovery of new cancer specific antigens on solid tumours is lagging behind, creating a huge bottleneck for the deployment of these potent therapies against (adeno)carcinomas.

The aberrant glycosylation of many different tumour types might be a key solution to this bottleneck, as glycobiologists have long realized. Mucin type *O*-glycosylation is often truncated on cancer cells, generating N-acetylgalactosamine(GalNAc)-α-Ser/Thr, called the Tn-antigen, or the α2,6-sialylated version thereof, (STn-antigen). Both are fairly specific for cancer cells and are virtually absent on most healthy cells. However, given the extreme potency of today's immunotherapeutic modalities, the combinatorial specificity of targeting both the truncated glycan moiety and the underlying peptide of a tumour cell-enriched protein would provide for enhanced safety. However, *O*-glycopeptides often have high micro- and microheterogeneity, many have low abundance within the proteome, and O-glycosidic bonds are labile in collision-induced mass spec fragmentation. This makes that these (S)Tn-antigens are very difficult to comprehensively map with the current gold-standard LC-MS/MS proteomics methodologies.

We have developed a novel LC-MS/MS based chemoenzymatic workflow, called TnMiner, that enables the very specific labelling of end-standing Gal and GalNAc-moieties of *O*-glycans with a tandem mass tag (TMT)-label. This TMT label allows for a powerful enrichment of the TMT-labelled *O*-glycopeptides via antiTMT-coated beads. It also boosts the ionization potency of these glycopeptides within the mass spectrometer, while also allowing for multiplexing up to six different tumour and healthy samples. This multiplexing strategy is crucial for relative quantification over the different healthy and tumour samples, in order to identify the Tn-glycopeptides that are only detectable within the tumour samples and absent in the healthy samples.

This TnMiner sample preparation is matched with a customized LC-MS/MS workflow for the quantitative profiling (TnProfiler) and identification (TnIdentifier) of these enriched (S)Tn-glycopeptides. Within the initial TnProfiler run, the mass spectrometer is only triggered to acquire a high resolution HCD spectrum, if the signature TMT-Tn-fragment ion is detected. In this way, the mass spectrometer's analysis time is dedicated for peptides that contain at least one Tn-antigen, ignoring the rest. Next to the TMT-reporter ions that are necessary for relative quantification, these HCD spectra are dominated by the TMT-Tn signature ion, as well as other TMT-labelled oxonium ions that originate from other *O*-glycans on the peptide that had end-standing Gal-moieties. This assists in identifying those events with just a single Tn modification, which we consider as prime targets for immunotherapy. We tested the TnProfiler workflow on a set of six different oestrogen receptor positive breast cancer cell lines and obtained 8313 trigger events, each one originating from a Tn-glycopeptide (estimated +/- 2000 different peptides), of which 5273 spectra had all six reporter ions present, meaning that these ions originated from a Tn-glycopeptide that is present in all six different cancer cell lines. This large number obtained from minute quantities of material illustrates the sensitivity of the method and hence its suitability for Tn-glycoproteome mapping of tumour biopsies. The information obtained from the TnProfiler run (i.e. relative quantification, retention time and mass) is used to generate a 'hitlist' for parallel reaction monitoring analysis (PRM) with ETD fragmentation in the subsequent TnIdentifier run, which is still being optimized. A first clinical target discovery study in breast cancer is ongoing

(P54) The Multifaceted Roles of SLC35A2 and SLC35A3 in Glycosylation and Cellular Homeostasis: A Global Transcriptomic and Proteomic Analysis

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The substrates for glycosylation reactions, namely nucleotide sugars, are predominantly synthesized in the cytosol. For these molecules to participate in glycosylation, they must be efficiently transported into the lumen of the Golgi apparatus or the endoplasmic reticulum (ER). This critical transport step is facilitated by specialized proteins belonging to the Solute Carrier 35 (SLC35) family.

Within this family, the SLC35A2 and SLC35A3 transporters have attracted significant attention due to their notable sequence similarity—sharing 53% of their amino acids—and their tendency to form complexes in living cells. Such complex formation suggests a potential cooperative mechanism in the regulation and execution of glycosylation. Our studies have demonstrated that the absence of the SLC35A2 transporter exerts a considerably greater detrimental effect on the glycosylation process compared to the absence of SLC35A3[1,2].

To investigate these dynamics more thoroughly, we conducted a global analysis to examine transcriptomic changes in cells deficient in either SLC35A2 or SLC35A3. Using RNA-Seq experiments, we identified significant alterations in gene expression profiles that not only highlighted the immediate impact on glycosylation pathways but also revealed compensatory responses by the cells. These transcriptomic data were complemented by a comprehensive proteomic analysis performed with LC/MS, which allowed us to quantify changes in protein expression. The proteomic findings were further validated and enriched by co-immunoprecipitation (Co-IP) experiments, which confirmed the physical interactions between the transporter complexes and other cellular components involved in glycosylation.

Moreover, we supported our molecular analyses with glycosylation profiling using lectins, which provided additional insight into how the disruption of these transporters alters the overall glycosylation pattern of the cell. The combined data from transcriptomic, proteomic, and glycosylation studies enabled a comprehensive evaluation of the functional consequences stemming from the loss of SLC35A2 or SLC35A3.

Our results revealed that the disruption of these transporters not only triggers the induction of epithelial-to-mesenchymal transition (EMT) but also activates compensatory mechanisms through the upregulation of alternative transporter proteins. In addition, we observed significant variations in the expression levels of various glycosyltransferases, further indicating that the loss of transporter function triggers widespread remodelling of the glycosylation machinery. Collectively, these findings shed light on the multifaceted roles of SLC35 transporters in regulating glycosylation pathways and maintaining cellular homeostasis, while also providing potential avenues for therapeutic intervention in diseases linked to glycosylation defects.

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(P55) Modelling the effect of knockout or overexpression of ARSK, the glucuronate 2-O-sulfatase

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The goal of this work is to establish an in vitro cell-based model of a mucopolysaccharidosis (MPS type X) that occurs due to arylsulfatase K (ARSK) deficiency, a rare monogenic lysosomal storage disorder with no treatment. Arylsulfatases are lysosomal enzymes that cleave sulphate esters in glycosaminoglycans, sulfolipids, or steroid sulphates, which have roles in cellular degradation, cell signalling, and hormone regulation. Loss of arylsulfatase activity has been linked to skeletal dysplasia, neurodevelopmental abnormalities, and other clinical phenotypes.

ARSK is the most recently reported member of this enzyme family in humans, and was found to catalyse removal of the 2-O-sulfate group from 2-sulfoglucuronate, which is found in heparan sulphate, chondroitin sulphate; such glucuronate-2-sulfatase activity has so far been found only in ARSK. We aim to achieve the following objectives; to establish a biochemical assay system for ARSK and clinically occurring mutants, to establish a cell-based genetic model of ARSK loss or overexpression in a relevant human cell line, and to understand the consequences of the above in a cellular and molecular context. In India, several clinical centres are specialized in rare disease diagnosis and management, but there is a significant need for preclinical research activity including capabilities in biochemical, cell-based, and animal studies. The larger goal of our work is to build these capabilities and facilitate clinical impact.

(P56) Characterization and profiling of menstrual cycle-dependent *N*-glycans in the human endometrium

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Infertility is a major health problem worldwide, and endometrial receptivity is critical for embryo implantation, a key step in pregnancy[1]. Glycosylation plays an important role in endometrial receptivity and implantation, and glycoconjugates, especially *N*-glycans, found on the surface of endometrial tissue play an effective role in cell-cell communication, maintenance of tissue structure, and regulation of the implantation[2]. Although the importance of glycosylation in embryo attachment and subsequent placental development is emphasized in the literature, the detailed *N*-glycan profile of endometrial tissue has not yet been fully elucidated. In this study, we aimed to characterize the *N*-glycome of human endometrial tissue using an in-gel block method, providing insights into glycosylation patterns associated with different phases of the menstrual cycle.

We successfully adapted and optimized an in-gel block method for extracting and analysing *N*-glycans from endometrial tissue samples, described previously[3]. This approach enabled purification of glycoproteins, efficient glycan release, followed by chromatographic separation and characterization. Using ultra-performance liquid chromatography, we revealed chromatograms showing glycan peaks from endometrial tissue samples collected at proliferative, early secretory, secretory, and late secretory menstrual cycle phases from healthy women. To further elucidate structural details, we performed exoglycosidase digestion assays, allowing for precise identification glycans and their structural motifs. These analyses contribute to establishment of a comprehensive glycan profile of the healthy endometrium as a blueprint for future studies.

Our findings provide a foundational glycosylation landscape for endometrial tissue, demonstrating the feasibility of using an optimized method for tissue samples for glycan release and characterization. Moving forward, we will extend this analysis to samples collected from four distinct phases of the menstrual cycle to investigate phase-specific glycosylation dynamics. This study sets the stage for deeper exploration into the role of glycosylation in endometrial function and potential implications for reproductive health, with a particular focus on infertility and subfertility.

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(P57) Single cell glycomic analysis of slide captured immune cells

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Peripheral Blood Mononuclear Cells (PBMCs), which include lymphocytes (such as T cells, B cells, and natural killer cells), monocytes, and dendritic cells, are crucial components of the immune system, contributing to both innate and adaptive immunity. These cells are primarily involved in recognizing and responding to pathogens, orchestrating immune signalling, and facilitating antigen presentation.

Due to their presence in circulating blood, PBMCs are easily isolated through standard blood collection, making them widely used in immunological research. PBMCs can reflect the systemic metabolic environment, serve as a proxy to understand the broader metabolic disturbances occurring in various metabolic diseases such as cancer, and bridge the gap between basic scientific discoveries and clinical applications.

We have recently developed a high-throughput antibody capture cell array-based slide using antibodies to CD4, CD8, CD19, and CD14 to determine the *N*-glycosylation patterns of each immune cell type in human PBMC isolates. Using PDMS stamps with patterned coordinates, a single cell capture version of the antibody slide array was created. Additionally, development of software programs to o identify regions containing a single captured cell and accurately record its location on the slide. Captured single cells were incubated with sprayed PNGase F to release *N*-glycans, followed by detection using a timsTOF fleX MALDI-QTOF mass spectrometer (Bruker, Billerica MA). Several thousand single cell N-glycome profiles of 40 structural compositions can be determined in 10 min or less.

Differential abundances of detected N-glycans can readily distinguish the different immune cell types. The array slide format is inherently flexible allowing for capture via antibodies, lectin or bait protein, and use of glycosidases and proteases for molecular profiling. It also allows sequential multimodal analysis workflows of the same captured cells, including detection of 250+ lipid species, 40+ *N*-glycans, 20+ glycogen derived glucose polymers, and 40+ tryptic peptides.

Using this tiered analysis, we were able to differentiate immune cell lineages, myeloid vs lymphoid and further cleanly distinguish CD4 from CD8 T-cells, CD19 B-cells and CD14 macrophages. This multimodal interrogation enhances our knowledge of the functional N-glycosylation patterns in the active immune system. Moreover, this approach has significant clinical implications, offering potential biomarkers for identifying patients who may benefit from targeted immunotherapeutic interventions, such as checkpoint inhibitors, monoclonal antibodies, or personalized vaccine strategies.

(P58) Deciphering Siglec ligands in cancer to improve immunotherapy

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Aberrant sialylation is a hallmark of cancer, contributing to immune evasion, metastasis and drug resistance[1]. Siglecs are a family of fifteen carbohydrate-binding proteins broadly expressed on the surface of immune cells[2-4] and have recently emerged as glyco-immune checkpoints as their sialylated ligands are upregulated on cancer cell surfaces[1,2,4]. The Siglec-sialic acid axis is a promising potential for immunotherapy and efforts are underway to develop therapies that block Siglec-sialic acid interactions[5-7]. However, there are major challenges associated with in determined precisely what are the specific carbohydrate ligands of Siglecs.

Carbohydrate ligands of Siglecs are difficult to study using conventional tools because (i) these interactions are biochemically weak and (ii) carbohydrate structures cannot be directly predicted from the genetic code. Advanced tools enhancing the avidity and stability of these interactions are needed to map Siglec-glycan landscapes on cancer cells.

These last years, the Macauley lab improved methods to detect Siglec ligands with the design of sensitive and quantitative methods and the use of recombinant soluble Siglecs[8-10]. Using these innovative tools in a flow cytometry-based application[11], we profiled Siglec ligands expression across cancer cell lines from the NCI-60 panel. We notably shown distinct Siglec binding patterns, providing insights into how cancer-specific sialylation signatures may influence Siglec interactions[8,9]. Accordingly, we got a 'fingerprint' of Siglec ligands on each type of cancer, which is currently used as a training dataset. Simultaneously, we are producing recombinant sialyltransferases to synthesize and study sialylated ligands in a controlled environment.

This currently facilitates the development of selective inhibitors of sialylation[12] and the biosynthesis of Siglec ligands on cell surface to validate sialyltransferases role in Siglec-sialic acid axis. We are combining the Siglec binding data generated with publicly available transcriptomics and proteomics data to predict the expression of what set of genes/proteins up regulate Siglec ligands. Overexpression and CRISPR-Cas9 knockout experiments[13] are currently developed to validate these predictions by assessing the impact of specific glyco-genes on Siglec ligand expression in cancer cells. From the predictive algorithms generated, we will conduct validation on clinical samples, particularly for patients not responding to current immunotherapies, which we hypothesize have higher levels of Siglec ligands.

This approach underscores the potential of targeting the Siglec-sialic acid axis to identify novel cancer biomarkers and improve personalized immunotherapy.

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(P59) Sialylation in haematopoiesis and leukaemia: transcriptional and glycomic insights

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Haematopoiesis is the lifelong process in which all blood cell types are generated from a pool of haematopoietic stem cells. Haematopoietic malignancies, as leukaemias, may arise in several cellular lineages and states of differentiation, giving rise to several subtypes of the disease. The pursuit of molecular and cellular mechanisms associated with leukaemia type and subtypes is still needed to better target these malignancies.

Glycosylation is a major post-translational mechanism characterised by the enzymatic addition of carbohydrate structures (glycans) to proteins and lipids of essentially all living cells. These molecular structures are composed of a set of several monosaccharides. The negatively charged sugar sialic acid is abundant in the outermost positions of glycans, playing a critical role in the interphase between cells and their external environment. While selected glycans are used as markers for pluripotency and stem cell differentiation, the analysis of sialoglycans in early haematopoiesis and leukaemia received episodic attention.

In this study, we probed sialyltransferase gene expression in single-cell RNA sequencing datasets of healthy and leukaemic samples and evaluated whether malignant transformation has a transcriptional reflection on these specific targets. Additionally, we performed a lectin-based sialoglycome profiling of a panel of leukemic cell lines, to match the discoveries made in silico.

Altogether, the malignancy-association of sialoglycans presence paves the way for new therapeutic targets.