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Cancer Biotech & ChemBiol

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Session 1 – Metabolism

Fluorescence quenched substrates for quantifying glucocerebrosidase activity within lysosomes.

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Loss of activity of the lysosomal glycosidase β-glucocerebrosidase (GCase) causes the lysosomal storage disease Gaucher Disease (GD) and has emerged as the greatest genetic risk factor for the development of both Parkinson Disease (PD) and Dementia with Lewy Bodies. There is significant interest into how GCase dysfunction contributes to these diseases, however, progress toward a full understanding is complicated by presence of endogenous cellular factors that influence lysosomal GCase activity. Indeed, such factors are thought to contribute to the high degree of variable penetrance of GBA mutations among patients. Robust methods to quantitatively measure GCase activity within lysosomes are therefore needed to advance research in this area, as well as to develop clinical assays to monitor disease progression and assess GCase-directed therapeutics. In this presentation we will summarize some of our recent work on the creation of selective fluorescence-quenched substrates that enable measuring the activities of endogenous levels of lysosomal GCase, and other lysosomal enzymes, within living cells. Among these substrates are those that can be used within live cells, but also those that can be used and subsequently fixed using simple methods. We show how the ability to apply these substrates to analyses of fixed cells permits simple multiplexed co-localization studies of GCase activity with subcellular protein markers. Furthermore, we illustrate how these substrates serve as sensitive tools for studying chemical or genetic perturbations of lysosomal enzyme activities using either fluorescence microscopy or flow cytometry. We show the quantitative nature of measurements made using these substrates with various cell types and demonstrate that they accurately report on both target engagement by GCase inhibitors and the GBA allele status of cells. Furthermore, through comparisons of GD, PD, and control patient derived tissues, we show there is a close correlation in the lysosomal GCase activity within monocytes, neuronal progenitor cells, and neurons. Accordingly, analysis of clinical blood samples using these substrates may provide a surrogate marker of lysosomal GCase activity in neuronal tissue.

O-GlcNAcylation, a new target for the early management of hemorrhagic shock.

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Shock is defined as an acute and critical heart failure leading to tissular hypoxia. Shock is the cause of one death every 2 seconds over the world. Most of the shock are distributive or hypovolemic shock. Unfortunately, the therapeutic solution to improve patient survival are very limited due to the complexity of the pathology and the multifactorial component leading to shock. Interestingly, these shock situation share common pathophysiological pathway such as hypoxia, inflammation and metabolic alteration.Over the past few years, we have been working on solution to reduce shock situation burden. We demonstrated that O-GlcNAc stimulation is beneficial during the acute phase of various shock (septic, hemorrhagic, anaphylactic).
However, metabolic differences between adult and child – specifically energy substrate availability, could impact the hexosamine biosynthetic pathway and the O-GlcNAc process.
Over a series of study, we have evaluated the potential impact of these metabolic difference on the young rat to evaluate the potential therapeutic interest of early O-GlcNAcylation during shock.

Overall, our data demonstrate the potential interest of such approaches in different condition and pathology. We are now developing new compound to be able to test this approach in more integrative models.
The hemorrhagic shock is an important cause of death worlwide with 1.9 million deaths each year. Contemporary resuscitation strategies as blood products, resuscitative fluids or vasopressors are symptomatic and inadequate towards this complex pathology. Thanks to different approach we have identified different protein of interest with peculiar impact of O-GlcNAc stimulation on them. For example, during the hemorrhagic shock the function of the Na/K ATPase pump which regulates the ionic balance, membrane potential and pH is altered. The O-GlcNAcylation is a dynamic post-translational modification involved in cell survival and stress response and its stimulation is associated with beneficial effects in ischemia-reperfusion and septic shock situation. Also, ACLY (ATP citrate lyase) is the only protein identified as less O-GlcNAcylated with a NButGT treatment (an OGA inhibitor which increase O-GlcNAc level in shock situation).

An overview of metal ion importance in glycosylation: the case of SLC10A7-CDG and TMEM165-CDG.

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The post-translational modification of protein by glycosylation is a complex and controlled enzymatic modification, involving hundreds of actors, including enzymes, transporters, chaperones and lectins.The great glycan structures diversity exposed at the cell surface, and generated within the secretory pathway of human cells is tightly regulated and controlled. The recent discovery of human congenital disorder of glycosylation (CDG) related to metal transporter defects opens a novel field of investigation, here referred as metalloglycobiology. How metals changes can affect the glycosylation machinery and hence the glycan structures produced? The goal of this presentation is to highlight the numerous steps and glycosylation pathway that involve metal-dependent actors, from the biosynthesis of nucleotide-sugars to

enzymatic reaction of glycosylation. This high metal-dependency in every glycosylation subtypes will be presented in regards to CDG cases affecting metal homeostasis, themselves impairing glycosylation. This is particularly the case of TMEM165-CDG. At the end, metal implication is often underestimated in glycobiology, although it is an absolute requirement for the proper establishment of glycosylation, and a potential regulator of glycan diversity.

How Sweet Is O-GlcNAc.

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The ventromedial hypothalamus (VMH) is known to regulate body weight and counter-regulatory response. However, how VMH neurons regulate lipid metabolism and energy balance remains unknown. O-linked β-D-N-acetylglucosamine modification (O-GlcNAcylation), catalyzed by O-GlcNAc transferase (OGT), is considered a cellular sensor of nutrients and hormones. Here we report that genetic ablation of OGT in VMH neurons inhibits neuronal excitability. Mice with VMH neuron-specific OGT deletion show rapid weight gain, increased adiposity, and reduced energy expenditure, without significant changes in food intake or physical activity. The obesity phenotype is associated with adipocyte hypertrophy and reduced lipolysis of white adipose tissues. In addition, OGT deletion in VMH neurons down-regulates the sympathetic activity and impairs the sympathetic innervation of white adipose tissues. These findings identify OGT in the VMH as a homeostatic setpoint that controls body weight and underscore the importance of the VMH in regulating lipid metabolism through white adipose tissue-specific innervation.

Regulation of Fatty Acid Synthase (FASN) by the nutrient-dependent modification *O*-GlcNAcylation.

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Fatty Acid Synthase (FASN) is a ubiquitous homodimeric and cytoplasmic enzyme producing fatty acids by using acetyl-CoA and malonyl-CoA as substrates, and NADPH,H+ as a co-substrate. FASN is the second enzyme of the de novo lipogenesis pathway. Fatty acids are stored in lipid droplets for energy storage but can also be used for the synthesis of second messengers, palmitoylation of proteins and membranes building (Raab and Lefebvre, 2022).
FASN is involved in cell proliferation and regulated by post-translational modifications (PTM) including *O*-β-N-acetyl-D-glucosaminylation or *O*-GlcNAcylation (Baldini et al., 2016). *O*-GlcNAcylation consists of the addition of a single *N*-acetyl-D-glucosamine or GlcNAc from UDP-GlcNAc on the hydroxyl group of serine and threonine residues by the *O*-GlcNAc Transferase (OGT). This modification is reversible as the *O*-GlcNAcase (OGA) hydrolyzes the GlcNAc residue. This PTM is nutrient-dependent as the levels of the nucleotide sugar UDP-GlcNAc depends on glucose, fatty acids, amino acids and nucleotides metabolism.

We have demonstrated that FASN and OGT are partners of interaction at the

hepatic level (Baldini et al., 2016 ; Raab et al., 2021). It has been shown that FASN *O*-GlcNAcylation in mice livers and in the HepG2 hepatic cancer cell line promoted its interaction with Ubiquitin-Specific Peptidase 2a (USP2a). Thus, ubiquitination of FASN is reduced resulting in a decrease of its proteasomal degradation and to an increase in its activity. In return, FASN inhibition reduces OGT expression and inversely OGT inhibition reduces FASN expression, which is reflected in a disturbance of cell cycle progression and a decrease in cancer cell viability.

At the pathological point of view, FASN is overexpressed in many cancers including hepatic cancer (Raab et al., 2022) where it promotes many hallmarks of cancer (Vanauberg et al., 2023). Due to its pro-oncogenic character, the study of the involvement of FASN *O*-GlcNAcylation in cancer is of particular interest. Wong and collaborators (2022) recently demonstrated that starvation of HeLa cells induced FASN *O*-GlcNAcylation and increased its activity, what promoted cancer cell survival. In the same way Groves and collaborators (2017) discovered that FASN was able to inhibit OGA during oxidative stress in U2OS osteosarcoma cells. OGA inhibition leads to an increase of the *O*-GlcNAcylation levels which could promote cancer cell survival.

From these data, we decided to go further on the study of the role of FASN *O*-GlcNAcylation in hepatic cancer. During my PhD, we aimed to identify FASN *O*-GlcNAcylated sites in HepG2 cells to decipher the role of each site on FASN expression, localization, activity, dimerization and interactions. At the cell level, we will check the effect on cell viability, proliferation, migration, invasion and on tumor growth. The global aim would be to inhibit FASN and OGT interaction as a new therapeutic strategy in hepatic cancer.

How to successfully involve patients and citizens in Glycoscience: learning from CDG and allies, the international community-centric research network for CDG.

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Introduction.

Patient and Public Involvement and Engagement (PPIE) is a growing field that recognizes the crucial role of patients and the public in the quality and relevance of healthcare services and research. Despite growing acknowledgment, implementation confronts challenges such imprecise role definitions, lack of readiness, and inadequate local-level advice. Funding organizations and publications require PPIE declarations, which may force academics to embrace them without interest or expertise. PPIE's importance in healthcare education is recognized, but concrete integration techniques are lacking.[1] In the branch of Glycobiology, knowing how complex sugars and proteins function together has direct implications for drug development and disease treatment.

Methods.

We performed a short systematic literature review to explore existing PPIE initiatives in the Glycobiology field. The databases chosen were PubMed and Google, with keywords such as “Patient and Public Involvement” and “Glycobiology”.

Results.

Our review revealed a huge absence of PPIE initiatives in Glycobiology. This highly contrasts with other healthcare areas such as oncology and mental health, where robust literature exists, detailing not just the benefits of PPIE but also providing frameworks for its effective implementation. These PPIE approaches have led to innovative solutions and fostered greater public

trust and engagement in healthcare research as people are more likely to trust scientific research if they are actively involved in the process.
Conclusion.
This research underscores the importance and feasibility of incorporating PPIE in the Glycobiology field.
There is an urgent unmet need to co-design approaches in Glycobiology, where patients are considered, partners starting from the research process. It is critical to provide a framework to effectively integrate PPIE into Glycobiology, enriching not only the scientific process but also ensuring that the outcomes are socially and clinically relevant for patients and their communities. Such a framework should include methodologies that allow researchers and their teams to systematically listen to and integrate patients' and citizens' voices. As the conversation around PPIE continues to evolve, it is imperative that Glycobiology research catch up, for the benefit of both the scientific community and millions of individuals affected by health issues related to glycans.

1: Karlsson, A. W., & Janssens, A. (2023). Patient and public involvement and engagement (PPIE) in healthcare education and thesis work: the first step towards PPIE knowledgeable healthcare professionals. BMJ Open, 13(1), e067588. https://doi.org/10.1136/bmjopen-2022-067588

Potential strategies for the treatment of PMM2-CDG: focus on pharmacochaperones.

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Mutations in the PMM2 gene cause phosphomannomutase 2 deficiencies, the most frequent congenital disorder of glycosylation (PMM2-CDG, MIM# 212065).
Mutant PMM2 leads to the reduced conversion of Man-6-P to Man-1-P, which results in low concentration of guanosine 5'-diphospho-D-mannose (GDP-Man), a nucleotide-activated sugar essential for the construction of protein oligosaccharide chains. To date, the only therapeutic options are preventive and symptomatic and the scientific community is putting its efforts in the development of a successful therapeutic strategy for this disabling disease. There are several ongoing researches for developing therapeutic strategies for PMM2-CDG; treatments based on increasing Man-1-P levels have been proposed, along with the administration of different mannose derivates, employing enzyme inhibitors or repurposed drugs to increase the synthesis of GDP-Man. A single repurposed drug that might alleviate a severe neurological symptom associated with the disorder is now in clinical use.
Proof of concept also exists regarding the use of pharmacological chaperones and/or proteostasis regulators to increase the concentration of hypomorphic PMM2 mutant proteins. Several of the patients are compound heterozygous of a severe pathogenic variant and a missense pathogenic variant that has been reported is associated with lowered PMM2 concentrations and reduced enzymatic activity.
Thus, the inherent instability described for most of the PMM2 mutants associated with PMM2-CDG led to the idea of testing small chemical compounds capable of improving protein folding in the cytoplasm (where PMM2 is

localized). Our group undertaken high-throughput screening and were identified a hit-to-lead compound that increased the thermal stability of several PMM2 mutations. In addition we have reported that the celastrol treatment led to a significant increase in mutant PMM2 protein concentration and activity that occur through Hsp90-driven modulation of the proteostasis network. Advancing the development of pharmaco-chaperoning, we have obtained different complete crystal structures of hPMM2 (free and bound to the essential activator glucose-1,6-bisphosphate (Glc-1 ,6-P2), three for the wild type and several for destabilizing mutations. Using these structures, we have proposed that ~80% and ~50% of the missense variants of the core and cap domains respectively are potential candidates for treatment with therapies that improve protein folding. The ongoing challenges facing the discovery of drugs to treat this orphan disease are focused on preclinical evaluation. To that end, we have generated cellular models using CRISPRcas9 gene editing and by differentiation of human iPSC to hepatocyte-like cells as targeted tissue for testing drug efficiency.

Session 2 - Infections & Immunology

The *Sporothrix schenckii* peptidorhamnomannan.

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Sporothrix species are the causative agents of sporotrichosis, the most reported cutaneous mycosis in Latin America. The *Sporothrix* cell wall (CW) is a highly dynamic structure that plays an important role in cell integrity and infection; and has a distinctive component that participates as a virulence factor and antigen, the peptidorhamnomannan (PRM).
The PRM is a glycoconjugate composed of rhamnose, mannose, glucuronic acid, and proteins, and although its carbohydrate moiety has been extensively studied, little was known about the protein core. We have identified 325 proteins in the *Sporothrix schenckii* PRM, many of which are cytoplasmic proteins with housekeeping functions, that could be acting as moonlighting proteins in the cell surface. Among the identified proteins, the GroEL/Hsp60 and 2 uncharacterized proteins, named Pap1 and Pap2, were selected for further analysis. The recombinant versions of the 3 proteins bound the extracellular matrix proteins laminin, elastin, fibrinogen, and fibronectin, and although the Hsp60 doesn't bind to type-I and type-II collagen, Pap1 and Pap2 do. Unlike Gp70, (the major adhesin described in *S. schenckii* and *Sporothrix brasiliensis*), which binds only to fibronectin and laminin, these PRM adhesins show a wider array of ligands. In addition, the recombinant Hsp60 and Pap1 protect the invertebrate model *Galleria mellonella* from a lethal *S. schenckii*

infection. Moreover, we demonstrated that preincubation of *S. schenckii* yeast with antibodies anti-rHsp60 or rPap1 before infection protected the larvae, probably by stimulating the insect immunological priming. This is the first report about moonlighting proteins in the *S. schenckii* CW with an important role during the pathogen-host interaction.

Rhamnose is not a glycan commonly found in medically relevant fungi, and its synthesis has only been reported in two human pathogens, *Sporothrix* spp. and *Pseudallescheria boydii*. In *Sporothrix*, it was shown that the CW rhamnose content correlates with virulence and that it's important for the CW composition and organization. We have proven that silencing of *RmlD*, an essential reductase for the synthesis of L-rhamnose, decreases the CW rhamnose content and increases

®-1,3-glucan levels, affecting not only

the CW composition, but also the interaction with the innate immune response. Also, *S. schenckii* rhamnose plays an important role during phagocytosis in a TLR4-dependent manner, since blocking of this receptor reduced WT yeast internalization by macrophages, but had no effect on *RmlD* silenced mutants, suggesting that TLR4 ligand is rhamnose. Finally, we demonstrated that rhamnose is important for *S. schenckii* virulence, since *RmlD* silencing decreased the fungus's ability to kill *G. mellonella* larvae, probably due to a loss of fungal adhesion to the host cells.

These results prove that the PRM is an essential component of the *S. schenckii* CW, participating in virulence and immune recognition.

***Botrytis cinerea* PMT4 is involved in *O*-glycosylation, cell wall organization, membrane integrity, adhesion and virulence.**

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Proteins found within the fungal cell wall usually contain both *N*- and *O*-oligosaccharides. *N*-glycosylation is the process where these oligosaccharides (hereinafter: glycans) are attached to asparagine residues, while in *O*-glycosylation the glycans are covalently bound to serine or threonine residues. The PMT family is classified into PMT1, PMT2 and PMT4 subfamilies, which are the key enzymes that specifically initiate protein *O*-mannosylation. By means of bioinformatics analysis within the *B. cinerea* genome database, orthologs to Pmt4 proteins from *Saccharomyces cerevisiae* and other fungal species have been identified. Based on these findings, we hypothesized that bcpmt4 is involved in *O*-glycosylation and plays an important role in the *Botrytis* protein glycosylation pathway. The expression of bcpmt4 gene into *Scpmt4*Δ or *Scpmt3*Δ mutants of *S. cerevisiae* restores glycan levels in the glycoprotein. The phenotypic analysis showed that *bcpmt4*Δ null mutants demonstrate a significant alteration in

hyphal cell wall composition, including a decrease of mannan levels and an increase in the amount of chitin and glucan. On the other hand, loss of bcpmt4 results in reduced glycosylation of the glycoprotein present in the *B. cinerea* cell wall. These changes were accompanied by a hypersensitivity to cell wall perturbing agents such as Congo red, Caffeine and Zymolyase or high hydrostatic pressure. Importantly, the *bcpmt4*Δ mutant showed reduced virulence, reduced biofilm formation and alterations in membrane integrity. These studies demonstrate for the first time that the *B. cinerea* bcpmt4 gene is involved in *O*-glycosylation and cell wall formation.

Glycan masking of H7 influenza virus HA head domain for epitope-focused immunogen design.

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Influenza virus rapidly mutates and escapes the immune system response. Antibodies targeting the viral surface glycoproteins are unable to recognize the antigen if mutations occurs at the binding interface. In particular, the creation of a glycosylation site and the addition of a sugar chain at the antibody interface can completely abrogate antibody binding. This phenomenon, referred as glycan masking, has been adopted in epitope-focused vaccine design to hide specific epitopes associated to lower therapeutic effects and to better expose sites of vulnerability to the immune system.
In this work, we applied the glycan masking technique to design immunogen candidates based on the H7 hemagglutinin head (H7-head) glycoprotein of influenza virus, with a particular focus on the Trimer Interface site II (TI-II) epitope, which is targeted by the protective human monoclonal antibody H7-200. We identified 25 sites on the H7-head, excluding the H7-200 epitope, which are highly prone to glycosylation. Each site is well exposed on the surface of the protein and contains one of the three residues forming the sequon for N-glycosylation NxS/T. We then combined the 25 glycosylation sites in 20 H7-head variants: ten immunogen candidates presented three extra glycosylation sites in addition to the native one (Tri mutants), seven presented five extra glycosylation sites (Penta mutants) and three presented seven extra glycosylation sites (Hepta mutants).
The Rosetta Suite, a program for protein modeling and design, was used to model the glycans and to predict changes in antibody binding for nine antigen-antibody pairs with available experimental structure (H7-200, m826, H7-235, FluA-20, H7.5, H7-167, L3A-44, L4A-14 and HNIgGA6). In particular, Rosetta was used to: 1) insert the sequon for N-glycosylation in the structure of H7-head; 2) model the 3-dimensional conformation of the glycan chains; 3) dock the panel of known antibodies at their respective binding site, and 4) predict changes in antibody binding for each antigen-antibody pair. A panel of 16 additional antibodies without known structure was also included in the experimental testing of the H7 head variants. The full panel of 24 antibodies covers the five major antigenic sites of

the H7 head domain: TI-II, Trimeric Interface I (TI-I), Site A, Receptor Binding Site (RBS) and Apex.
Of the 20 candidates, only ten expressed well as recombinant proteins and all of them bound to H7-200, as predicted by Rosetta. Binding was also retained for the antibodies targeting the Trimer Interface site I (TI-I), while partial or complete loss of binding was shown for antibodies targeting the receptor binding site (RBS), the Apex and the Site A epitopes. Finally, we identified two different mechanisms of binding abrogation mediated by the glycosylations.

_N_-glycans as key immune-checkpoints at the frontiers of inflammation, autoimmunity and cancer.

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The immune system is governed by a series of stimulatory and inhibitory pathways in which the disruption of the control of these molecular checks can lead to unpredictable autoimmune or cancer states. The mechanisms underlying the genesis of the loss of immunological tolerance in autoimmunity or the creation of immunosuppressive networks in cancer are still elusive. Glycans have been highlighted as essential determinants that integrate the regulatory networks that guide both innate and adaptive immune responses. Changes in protein glycosylation are a hallmark of immune-mediated diseases, in which glycans act as master regulators of the inflammatory response being fundamental molecular determinants for the discrimination between “self”/“non-self” (*Alves, FEBS Lett 2022*). Our results in Systemic Lupus Erythematosus (SLE), a classical autoimmune disease, revealed a unique glycan signature characterized by an increased abundance and spatial distribution of unusual mannose-enriched glycans. This abnormal exposure of mannosylated glycans at the surface of kidney cells from patients with lupus nephritis (LN) was shown to promote an increased recognition by specific glycans-recognizing receptors, expressed by immune cells, potentially contributing to the immunopathogenesis of Lupus. This abnormal glycosignature of LN was demonstrated to be due to a deficient complex N-glycosylation and a proficient O-mannosylation pathway. Importantly, from the clinical standpoint, levels of mannosylation detected in kidney biopsies from LN patients at diagnosis were demonstrated to predict the development of chronic kidney disease (CKD) with 93% of specificity (*Alves I, et al. Arthritis and Rheumatology 2021*). In line with this, our results on Inflammatory Bowel Disease (IBD) also point towards a role for complex N-glycans in the immunopathogenesis of IBD (*Dias A, et al. PNAS 2018; Verhelst X, et al. Gastroenterology 2020*)
At the other pole of the immune response, in a cancer context, where immunosuppressive networks promote cancer progression, we also demonstrated the immune-regulatory properties of glycans. We showed that complex branched N-glycans structures, typically overexpressed by cancer cells, are used by colorectal tumor cells to escape immune recognition, by instructing the creation of immunosuppressive pathways through inhibition of IFNγ production. The removal of this “glycan-mask” was found to expose immunogenic glycans that potentiate immune recognition through

DC-SIGN-expressing immune cells resulting in an effective anti-tumor immune response (*Silva M & Fernandes A, et al. Cancer Immunology Research 2020*). In summary, our results demonstrate the regulatory power of glycans in governing both innate and adaptive immune responses with important roles in the pathogenesis of major diseases such as cancer and autoimmunity, pinpointing glycans as key checkpoints with promising clinical and therapeutic applications in autoimmune diseases and cancer.

Surface carbohydrates of *Echinococcus* and host lectins.

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The larval stages of cestode parasites belonging to the genus *Echinococcus* are important causes of disease in humans and domestic ungulates. The species cluster *E. granulosus sensu lato* causes cystic echinococcosis, characterized by bladder-like structures that develop within various organs. *E. multilocularis* causes alveolar echinococcosis, characterized by parasite masses that invade the liver parenchyma. Throughout the genus, the defining feature of these larvae is the so called laminated layer (LL). This is an acellular coat that protects parasite cells from direct attack by host immune cells. Our work of over 20 years showed that the major component of the LL are mucins and that the corresponding. glycans are based on the conventional cores 1 and 2, decorated or not. In *E. granulosus sensu lato*, the core Gal residue can be decorated with one or more Galβ1–3 units, and the resulting Galβ1–3 chain can be capped by Galα1-4; the Galβ1–3 residues can also bear Galα1-4Galβ1–4GlcNAcβ1-6 ramifications. In addition, the core 2 GlcNAc residue can be decorated by Galα1-4Galβ1–4. In *E. multilocularis*, the overall picture is similar but elongation with Galβ1–3 is absent and the core Gal residue can be directly capped.

Larval growth is accompanied by the shedding of abundant LL particles into the host's internal milieu, as shown by T. Barth's group (Ulm, Germany) in human infections. This calls the question of how the host deals with these materials. In an *in vitro* screening using 36 recombinant mammalian innate lectins, only Clec4F bound to the LL mucins and their glycans. Clec4F is a cell surface C-type lectin with GalNac/Gal specificity. In rodents, Clec4F is expressed only in Kupffer cells (KC), the liver macrophages exposed to the vascular space. Our recent works shows that LL mucins injected into mice circulate and are captured selectively by KC, mostly in Clec4F-dependent manner. Also, in mice experimentally infected with *E. granulosus*, LL mucins are taken up in Clec4F-dependent fashion by KC, in addition to being found in cells at the infection site (peritoneal cavity). Mice deficient in Clec4F tend to develop lower parasite burdens and express (during chronic infection) lower levels of the suppressive molecule PD-L1 in KC than wild-type mice.

Larval *Echinococcus* parasites are thought to have dwelled in the liver of rodents ancestrally, and rodent liver is the host/organ preference of *E. multilocularis*. KC are known to play roles in immune tolerance. We thus believe that the interaction between the LL glycans and Clec4F is an evolutionary adaptation contributing to immune regulation by larval *Echinococcus*. Clec4F is probably an important player in natural *E. granulosus sensu lato* infections in ungulates, which express Clec4F in both liver and lymphoid organs. Humans do not

express functional Clec4F: we are analyzing the distribution of LL materials in infected Clec4F-deficient mice, in search of clues on the biology of human cystic echinococcosis.

trans-Sialidase and Virulence in _Trypanosoma cruzi_: Insights into Immune Modulation and Membrane Protein Trafficking.

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Trypanosoma cruzi, the protozoan agent of Chagas disease (American trypanosomiasis) is unable to synthesize sialic acids *de novo*. However, the sialyl residue is required to interact with the host cell and to evade lysis by humoral factors. To compensate for this deficiency, the parasite expresses an enzyme called *trans*-sialidase (TS) that is capable of transferring terminal alpha 2,3-linked sialyl residues between macromolecules, resulting in the sialylation of their surface mucins. Both, TS and mucins are shed in microvesicles that bud from the parasite surface and then their GPI anchorages are cleaved by blood phospholipases. Since TS has evolved to persist in the blood, the modification of the sialylation pattern also occurs in cells of the mammalian host that are distant from the infection foci. The altered sialylation of immune cells modifies the peripheral immune responses. TS also induces apoptosis of male mouse thymocytes. Additionally, the enzyme is capable of desialylating platelets and red blood cells reducing their half-life. The parasite also expresses an “inactive” isoform of TS. Its catalytic Tyr 342 is replaced by His bringing the enzymatic activity to residual values while retaining its ability to recognize the substrate (lectin activity). This isoform is involved in the attachment to the host cell and its codifying genes are found in the most aggressive parasite lineages. Therefore, TSs are considered outstanding virulence factors due to their effect on infectivity and survival of the parasite in blood, as well as the changes induced in the immune response.

Recent studies on TS allows to understand the trafficking pathways and surface disposition of membrane proteins in this parasite. By following the *de novo* synthesis of TS during the transition from intracellular amastigotes to trypomastigotes, we detected the involvement of the contractile vacuole, an organelle that is absent in mammals, in the process. On the parasite surface, TS is GPI-anchored in domains that are separate from the mucin domains, which serve as the primary acceptor substrate for the sialyl residue. The spatial distance between these domains prevents the surface TS directly sialylating mucins. Instead, this activity is carried out by the shed enzyme, which transfers the sialyl residue from host’s cell glycoproteins located near the infection focus. Furthermore, this specific organization of domains appears to be governed by the GPI acceptor sequence.

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FLASH PRESENTATIONS

Glycosylation patterns of hemocytes from *Cherax quadricarinatus* after immunostimulation.

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Glycan structure arrangement in hemocytes from the Crayfish (*Cherax quadricarinatus*) has been proposed to play relevant role in hemocytes activation. In this work we have identified the glycophenotype of hemocyte subpopulations using lectins in basal and after *in vivo* stimulation with *V. parahaemolyticus* N16 (Vp, 1×10^9 cells/mL), *E. coli* BL21A1 (Ec,1×10^9 cells/mL), or microparticles of rice glucans (MPA, 200 ng/mL). By flow cytometry was characterized subpopulations of hemocytes and measured viability, phagocytosis activity, ROS production, and carbohydrates in the cell surface by lectins (ALL, VVA, ConA, MAA-I, GS-I, GS-II and SBA-I). The results showed increased 84.7% ROS production after MPA stimulation, 79.3% with Vp or 89.3% with Ec after 2-4 h stimulation compared with (50.3%) control. Phagocytosis activity also increased 43.3% after MPA, (45.1%), Vp or (35.5%) Ec treatments. Main glycan expression in hemocytes showed that the MPA treated groups increased (85.5%) α-2,3 NeuAc and 53.6% GalNAc; after Vp 65.3% of α-2,3NeuAc and 56.3% of GlcNAc; after Ec treatment increased GlcNAc (46.7%) and GalNAc (61.7%) when compared with basal and control group. The immunostimulants seems to be useful to identify differential activation process and showed to be useful to characterize the composition of glycans per population of hemocytes.

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Proteins and glycoproteins from the anterior midgut of *Meccus pallidipennis.*

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Chagas disease is caused by the protozoan *Trypanosoma cruzi.* The main transmission mechanism for the parasite in endemic areas is contact with the feces of infected triatomine bugs. Part of the life cycle of *T. cruzi* occurs in the digestive tract of triatomines, where vectors and parasites engage in close interaction at a proteomic-molecular level. The aim of the present work was to identify differences in protein and glycoprotein expression patterns of the anterior midguts of fifth instar nymphs and female adults of *Meccus pallidipennis*. Protein profiles of electrophoretic separations were obtained using commercial precast minigels, and glycoproteins were revealed by Lectin blots with Concanavalin A (Con A), Wheat Germ Agglutinin (WGA), and Peanut agglutinin (PNA). The electrophoretic analysis of nymphs and females detected 32 bands in both cases. Banding patterns featured three unique bands of different molecular weights. Abundant mannose residues were detected in both nymphs and females, with 24 and 23 bands respectively. Sialic acid and N-acetylglucosamine residues were also abundant, with 19 shared bands. However, unlike in adults, a large number of high molecular weight bands were observed in the nymphs' midgut. Identification of galactose and N-acetylgalactosamine residues, characteristic of O-glycosylation in both samples, was lower than with the other two lectins, with only 4 shared bands. In females, 12 recognitions stand out, of which 8 are exclusive to this stage. The protein profile between nymphs and females is very similar; however, some differences are observed in the glycosylation patterns. Differences in protein glycosylation patterns may indicate some specificity of functions in the proteins of each stage of development. Studies of glycoproteins at the intestinal level of the Chagas disease vector are important because parasites have carbohydrate-binding proteins that recognize glycosylations at the intestinal tract level. These become important targets in order to interrupt the *T. cruzi* biological cycle.

Session 3 – Cancer

Harnessing the Power of Sugars in Cancer: Advancing Therapeutic Approaches through Glycoimmunology.

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Cancer often leads to impaired immune function and unfavourable outcomes. However, this scenario also presents an opportunity to reveal strategies that can harness the immune response and improve patient outcomes. In triple negative breast cancer (TNBC) we had

identified sialyl Tn (STn) and Sialyl Lewis X (SLeX) as related to immunosuppression and biomarkers for stratification.
The STn is a truncated O-glycan associated with poor prognosis in cancer. Within TNBC, patients expressing STn exhibit reduced survival rates. Analysis of genetic databases of TNBC patients revealed transcriptome alterations linked to the ST6GALNAC1 gene, which is associated with proliferation and immune processes such as leukocyte migration and response to chemokines.
In addition, *ST6GALNAC1* correlates positively with the infiltration of regulatory T cells and M2 macrophages, both associated with a pro-tumoral and immunosuppressive environment. Anti-STn monoclonal antibodies (mAb) have
raised several constraints due to their low affinity and specificity. To overcome this, we developed a platform for high specific and affinity mAbs that react with higher intensity to different cancers, but not to healthy tissues. In a preclinical breast cancer model, our anti-STn mAb successfully overcame immunosuppression and enhanced immune response, indicating the potential of STn blockade as an effective therapeutic approach.
SLeX was found to be expressed in all TNBC patients, but the subgroup with higher SLeX expression showed lower levels of cytokeratins. The downregulation of specific cytokeratins may impact immune recognition and contribute to immune evasion by cancer cells. This talk will present the latest research findings on the biology of STn and SLeX, as well as immune-related strategies employed to enhance anti-tumor immune responses. Additionally, we will discuss other glycans, particularly sialylation, which have attracted interest due to their immunoregulatory roles and potential as targets for immunotherapy.

Aberrant glycosylation in gastric cancer: novel molecular mechanisms controlling disease progression.

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Gastric cancer is a silent disease often diagnosed at advanced stages, being among the most common and lethal cancers worldwide. Paramount evidence shows that post-translation alterations, such as glycosylation, hold a significant relevance on cancer biology (1). Glycoconjugates are essential players in the tumour microenvironment, with pivotal roles in extracellular matrix shaping and cancer cell communication (2). Particularly, glycosaminoglycans (GAGs) have been show to play important roles in cancer cell signalling with functional effects on tumour progression and therapy response (3).
Gastric clinical samples profiling revealed specific GAGosylation signatures along the gastric carcinogenesis cascade. To functionally address the role of specific glycosylation profiles, we established glycoengineered gastric cancer cell models with defined GAGosylation, either by modulating the glycosyltransferases involved in HS biosynthetic pathway or the expression of Syndecan-4 (SDC4), a main HS carrier. We showed that increased HS expression concomitant with upregulation of SDC4, promoted a more aggressive cancer phenotype, characterized by higher cellular motility

and invasion, and altered receptor tyrosine kinase activation (4). Moreover, we demonstrated that HS-glycosylated SDC4 is packed on extracellular vesicles (EVs) secreted by cancer cells. Noteworthy, SDC4 impacted EV uptake by recipient cells and modulated the organ tropism of cancer EVs to the gastric cancer metastatic sites (5).
Our data discloses previously unappreciated roles of HS GAGs in gastric cancer biology and unveils their potential as tumour biomarkers but also as therapeutic targets to block cancer cell signalling and communication.

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Deciphering the role of O-GlcNAcylation of TATA-Box Binding Protein (TBP) in signaling pathways associated with colorectal cancer.

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For several decades, a plethora of studies have established a close link between eating habits and the occurrence of some cancers. Indeed, western diets, obesity, and diabetes are correlated with an increased risk of developing certain cancers. In the very early stages of initiation and development of cancer, alteration of gene expression is a well-known phenomenon. In eukaryotes, gene expression is performed by three RNA polymerases that request a variety of molecular complexes to recognize promoters, form the pre-initiation complex (PIC), and initiate transcription. Among the fast variety of PIC, the TATA-box binding protein (TBP) is the sole common factor. TBP has been long seen as a mere scaffold for the assembly of the PIC but recent data highlighted its role in a nutrient-dependent manner regulation of transcription and cellular metabolism. TBP is indeed post-translationally modified by the nutrient sensor O-GlcNAcylation. This dynamic modification is catalyzed by a unique couple of enzymes: O-GlcNAc Transferase (OGT) which transfers the GlcNAc moiety from UDP-GlcNAc and *O*-GlcNAcase (OGA) removes it. OGT activity is dependent on the concentration of UDP-GlcNAc, the end-product of the hexosamine biosynthetic pathway (HBP). The synthesis of the UDP-GlcNAc requires metabolites from all major cellular metabolic pathways and shortage or increase in one of these pathways impacts the flux through the HBP and alters the UDP-GlcNAc level. Our previous data showed that TBP is targeted by *O*-GlcNAcylation and its

cycle at the T114 residue affects its dynamic interaction with promoters and alters gene expression associated with lipid metabolism (Hardivillé et al., 2019). We identified two other *O*-GlcNAcylation sites on TBP at T126 and S158 residues but the role(s) of their O-GlcNAcylation on the transcription machinery remain(s) to uncover. Here, we endeavor a comprehensive study of the *O*-GlcNAcylation of TBP at T114 and S158 using multi-omics approaches. We first developed cell lines where each site was targeted for gene edition using CRISPR/Cas9 technology. The genome of HeLa cells was edited to mutate T126 or S158 residues into Alanine (T126A or S158A), hence invalidating their *O*-GlcNAcylation. Our results showed that we succeed to obtain a homozygote mutant for the S158 *O*-GlcNAcylation site but not for the S126 site. We then studied the assembly of pre-initiation complexes for the different TBP *O*-GlcNAcylation mutants. To do this, endogenous *O*-GlcNAcylation site edited mutants of TBP were immunoprecipitated and interaction partners were identified and quantified by proteomic approaches. *O*-GlcNAcylation of TBP impacted the assembly of alternative PICs which could be reflected in the transcriptome and cellular physiology. To investigate this point, we finally performed RNAseq analyses on the different TBP-*O*-GlcNAc mutants to highlight altered gene networks and the associated biological pathways.

Taken together, these data should enable us to better understand the regulation of TBP on gene transcription by the “nutrient sensor” O-GlcNAcylation and update our knowledge of the basal transcription machinery and its pathophysiological involvement in the etiology of metabolic-related disorders such as cancer. Abnormal hyperglycemia-increased *O*-GlcNAcylation of TBP may promote the development of precancerous lesions through increased stimulation of genes involved in cell proliferation, notably those whose promoters possess a TATA box element.

Elucidating the role of FUT1&2 alpha1,2 fucosyltransferases in head and neck squamous cell carcinoma.

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Head and neck cancer is the seventh most common cancer in the world, claiming ~300,000 deaths yearly. Most head and neck squamous cell carcinomas (HNSCC) arise from the mucosal epithelium in the oral cavity, pharynx and larynx. The α(1,2)-fucosyltransferases FUT1 and FUT2, despite their similar catalytic activity, have drastically different roles in cancer, likely driven by differences in substrate specificity. FUT1 is associated with poor prognosis, and drives self-renewal/stemness, migration, invasion, adhesion, and drug resistance in multiple cancer types. In contrast, FUT2 expression is associated with increased survivorship in HNSCC. The goal of our research is to understand the roles of FUT1&2 in HNSCC.

To explore the potential contribution(s) of FUT1&2 to aggressive tumor characteristics in HNSCC, we have employed tissue immunohistochemistry of human HNSCC samples, CRISPR-editing of established HNSCC cell lines, flow cytometry, mass spectrometry, transcriptomics, and immunoblotting of HNSCC cell line lysates. Here, we document differential surface expression of α1,2-fucosylated epitopes in a panel of normal, dysplastic, and HNSCC cell lines, identify a set of

potentially α1,2-fucosylated signaling and adhesion molecules including EGFR, CD44, and integrins via nLC-MS/MS, and present evidence that EGFR is among the α1,2-fucosylated and LeY-displaying proteins in HNSCC. Furthermore, CRISPR-mediated knockout of FUT1 dramatically reduces the surface expression of α(1,2)-fucosylated epitopes. The observed reduction in FUT1-mediated α(1,2)-fucosylation broadly impacts the phosphorylation of multiple receptor tyrosine kinases (RTKs) and downstream signaling targets, including but not limited to, EGFR-signaling pathways. In contrast, FUT2 overexpression reduces EGFR ligand-dependent phosphorylation and restores regulation of cell signaling and adhesion, leading to reinforcement of epithelial cell characteristics.

Given that surface receptors that govern critical cellular signaling cascades are highly modified by N- and O-linked glycans, we propose that α(1,2)-fucosylated glycans alter the activity of these molecules, leading to the dysregulation of cell signaling in HNSCC, thereby thus serving as critical modulators of HNSCC carcinogenesis.

Deciphering aberrant glycosylation in colorectal cancer.

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Metabolic reprogramming is widely observed in malignant tumors, including colorectal cancer (CRC). Tumor cells have a high rate of glucose uptake and part of their metabolism supplies the hexosamine biosynthesis pathway, whose exacerbation may favor changes in protein glycosylation, as its final product, the activated monosaccharide UDP-GlcNAc, is crucial for glycan biosynthesis. Aberrant glycosylation affects different cellular and molecular mechanisms related to the malignant phenotype. For example, increased levels of β1,6-GlcNAc branched N-glycans (a structure that is catalyzed by the N-acetylglucosaminyltransferase V, MGAT5 or GnT-V) are associated with the acquisition of migratory phenotype and the loss of apical junctional complex stability. In this lecture, recent advances related to metabolic alterations and protein glycosylation in the context of CRC will be presented.

Deciphering the context-dependent roles of mannose metabolism in cancer.

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In mammals, normal biosynthesis and catabolism of mannose is essential for life. In the mannose metabolism, mannose phosphate isomerase (MPI) plays a key role by catalyzing interconversion between fructose 6-phosphate (F6P) and mannose 6-phosphate (M6P). The conversion from F6P to M6P by MPI is essential to synthesize GDP-mannose from glucose for building up dolichol-linked oligosaccharides (DLOs) for N-glycosylation in the endoplasmic

reticulum, as well as for preventing the premature degradation of DLOs by glycan quality control system1,2,3. In contrast, the conversion from M6P to F6P by the same enzyme is critical to drain the excess M6P to glycolysis, while the cellular function of this metabolic pathway has long remained unknown, except that inadequate catabolism of mannose via glycolysis inhibits glucose metabolism.

A high dose of mannose is known to inhibits cell proliferation and enhances chemotherapy, particularly in cancer cells that express low levels of MPI4. In the present study, we established MPI-KO human cancer cells to explore the key mechanism behind the anti-cancer activity of mannose. Our findings indicated that the large influx of mannose exceeding the capacity to metabolize it caused ATP insufficiency and the loss of deoxyribonucleoside triphosphates5. These cells were cycling extremely slowly, and upon encountering replication stress, they were unable to rescue stalled forks via dormant origins, thus exacerbating replication stress. These findings suggest that the conversion of Man-6-P to Fruc-6-P mediated by MPI serves as a guardian of genome through the maintenance of metabolic integrity.

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The Sd^a antigen synthase B4GALNT2 in cancer.

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The carbohydrate antigen Sd^a consists of a α2,3-sialylated chain to which a GalNAc residue is β1,4-linked to the subterminal Gal. Its last biosynthetic step is catalyzed by the GalNAc transferase B4GALNT2, which is expressed at a very high level by normal colon while it is variably down-regulated in colorectal cancer (CRC). According to "The Cancer Genome Atlas" (TCGA), patients with higher B4GALNT2 in CRC display a much longer overall survival and a gene signature associated with lower malignancy (1). The bases of B4GALNT2 down-regulation in CRC are multi-factorial and likely include lower DNA methylation of an "open sea" site between exons 6 and 7 and high expression of miR-204-5p (2). The biosynthesis of Sd^a and that of the cancer-associated sialyl Lewisx (sLex) antigen are mutually exclusive because the two structures derive from different substitutions (with GalNAc or Fuc) of the same α2,3-sialylated type 2 chain acceptor. To investigate whether high B4GALNT2 and lower malignancy were linked by a causal relationship, B4GALNT2 cDNA was constitutively expressed in the CRC cell lines LS174T, SW480 and SW620 (3). In the first, which spontaneously expresses sLex, B4GALNT2 induced Sd^a expression and a concomitant decrease of sLex. In SW480 and SW620, which lack both antigens, besides Sd^a-expressing variants by B4GALNT2 transfection, sLex-expressing variants were also generated by fucosyltransferase 6 (FUT6) transfection. Thus, the impact of Sd^a expression was investigated both in relation to sLex inhibition (LS174T) or independenty (SW480 and SW620). Cell

lines SW480 and SW620 are closely related, being derived from the primary tumor (SW480) or from a lymph node metastasis (SW620) of the same patient. Microarray analysis indicated that in these two cell lines B4GALNT2 expression consistently modulated 128 genes, while FUT6 expression modulated 1779 genes. Despite the close relationship between SW480 and SW620, several genes were modulated by glycosyltransferase expression in either cell line, but not in both. However, if LS174T were included in the statistical analysis, we found that only a few genes displayed consistent B4GALNT2-induced modulation in the three cell lines. Of these, the most relevant appears to be the down-regulation of the colon cancer-promoting gene SPON2. Functional analysis of the three cell lines indicated that B4GALNT2 induced a general attenuation of the phenotypic features associated with stemness. Collectively, current data indicate that B4GALNT2 is causally involved in attenuation of the malignant phenotype in CRC, providing a rationale for the observed better outcome of the high B4GALNT2-expressing patients.

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A genetic entry point to harness the untapped potential in glycobiology from decoding functions to cancer specific targeting.

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Breakthroughs in O-glycobiology have gone hand in hand with the development of new technologies, such as advancements in mass spectrometry and the facilitation of genetic engineering in mammalian cell lines. High-throughput glycoproteomics has enabled us to draw a comprehensive map of O-glycosylation, and mining this information has supported the discovery of functions related to site-specific O-glycans.

Among the important next challenges will be to define the contextual functions of glycans in cellular differentiation and transformation. We have used a genetic entry point and targeted specific glycogenes to generate a library of cell and tissue models that selectively differ in their capacity to produce specific glycan structures. The engineered cell and tissue libraries have been used to show how specific truncated cancer-associated O-glycans, such as Tn and STn, impact tissue homeostasis epithelial transformation and how they interact with glycan-binding proteins, promoting an immuno-suppressive environment.
These findings explain why Tn and STn are widespread in epithelial cancers, serving as promising immunological targets for treating human carcinomas of epithelial origin. By analyzing O-glycoproteomics data we have designed a large set of glycopeptide targets and developed corresponding high-affinity monoclonal antibodies with exquisite cancer selectivity and target-specific cytotoxicity both in vitro and in vivo as CAR T cells and as antibody-drug conjugates.

_O_-GlcNAcylation in cancer: The establishment of the malignant phenotype in cancer stem cells.

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Colorectal carcinoma (CRC) is a leading cause of cancer mortality. Although the origin of cancer has not been totally identified, tumor growth and metastasis can be promoted by a small sub-population of cancer cells called cancer stem cells (CSC). These CSCs populations are characterized with an increased tumor initiation capacity, self-renewal and differentiation, metastasis and resistance to chemo and radiation therapies. The generation of CSCs is probably connected to genetic changes in components of signaling pathways which control self-renewal and pluripotency and then drive functions and phenotypes of CSCs. As a result of accumulated genetic and epigenetic alterations and also complex interconnection with the tumor microenvironment (TME), CSCs can evolve and convert to full malignant cells. One of the main pathways in CSCs that connects the alteration in TME and signaling network in the establishment of CSCs malignancy is the hexosamine biosynthetic pathway (HBP). The end product of HBP, UDP-N-acetylglucosamine (UDP-GlcNAc) is utilized by the enzyme *O*-GlcNAc Transferase (OGT) for the glycosylation of proteins at Ser/Thr residues, a processed named O-GlcNAcylation. This dynamic posttranslational modification has emerged as a key regulator of diverse cellular processes including several hallmarks of cancer. Recent studies have begun to connect OGT and *O*-GlcNAcylation to the regulation of CSCs, however, their role has not been fully described. To elucidate the role of *O*-GlcNAcylation in the establishment of the malignant phenotype in CSCs, we chose the following models of CRC: the SW480 and SW620 cell lines which represent primary and metastatic tumors, respectively. According to the flow cytometry analysis, SW480 cell line only expresses the marker of stemness CD44 on the cell membrane meanwhile SW620 only expresses CD133. Following pharmacological inhibition of OGT - but not *O*-GlcNAcase (OGA) the antagonistic enzyme of OGT that removes *O*-GlcNAc residues - to modulate *O*-GlcNAcylation levels, we found an increased in the expression of both stem cell markers in our colon malignant cell lines, and the appearance of a double positive (CD44+/CD133+) small stem cell-like subpopulation (which corresponded to 1–10% of total cells). This population, in comparison with the single-marker population, displayed a more aggressive malignant phenotype measured by the increased in clonogenicity and spheroid formation abilities in 3D culture. Although, we described that the modification of *O*-GlcNAcylation induced a more malignant phenotype in the CSCs, further studies are required to clarify the exact mechanism.

FLASH PRESENTATIONS

Role of glycosylation in thrombocytopenia and acute myeloid malignancies associated with RUNX1 Familial Platelet Disorder.

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Congenital disorders of glycosylation (CDG) are frequently associated with problems in the coagulation process, highlighting the importance of this modification for hemostasis. This observation opens the possibility of other congenital disorders presenting as hematological deficiencies that could be related to glycosylation defects but that are still not understood. In this project, we focus on glycosylation defects in RUNX1 familial platelet disorder (RUNX1-FPD), a hereditary condition that affects platelet production and that is a predisposition to leukemia development. RUNX1 is a transcription factor related to hematopoiesis, which has been implicated in the orchestration of sialyltransferase expression. Therefore, we hypothesize that a hyposialylation state is part of the mechanism involved in RUNX1-FPD. The general aim of this project is to explore if RUNX1-FPD is related to glycosylation defects. To address this, we generated a RUNX1 knockout K562 cell line, and the preliminary data indicated that these cells have a decrease of α2,3 sialylation but the same level of α2,6 sialylation. These data agree with the literature reports indicating RUNX1 as an important orchestrator of sialyltransferases involved in adding sialic acid at position α2,3. The following steps of the project include analysis of sialylation levels using DMB derivatization, selective periodate oxidation and aniline-catalyzed oxime ligation method (PAL) to confirm the sialylation levels. Furthermore, we will use a 2,3 sialidase to explore the effects of decreasing this specific type of sialylation on myeloid cells. In the end, we will investigate whether changes in myeloid cell glycosylation affect recognition by Siglecs. The results of this project will expand the knowledge available regarding the function of glycosylation in RUNX1-FPD, an unexplored perspective of this disease, and explore the role of sialylation in hemostasis and leukemia development.

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Golgi Phosphoprotein 3 mediates the association between β3GalT-IV and ST3Gal-II and the subcellular localization of ST8Sia-I glycosyltransferases.

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Gangliosides are sialic acid-containing glycosphingolipids expressed primarily on the outer leaflet of the plasma membrane of eukaryotic cells. Glycolipid glycosylation is an intricate process that mainly takes place in the Golgi by the complex interplay between glycosyltransferases. In spite of the advance in our knowledge about the formation of glycolipid glycosyltransferases complexes, the mechanisms regulating glycosylation in both health and disease remain unclear. Golgi phosphoprotein 3 (GOLPH3) was the first Golgi-associated oncoprotein to be reported. Despite its importance in cancer biogenesis, the role of the Golgi oncoprotein GOLPH3 in the regulation of glycolipid metabolism remains an underexplored area of research which only recently gained attention. In the present work we show that downregulation of GOLPH3 in a glioblastoma cell line produces a reduction of GD1a ganglioside and an increase of GM1 ganglioside. Similar results were also obtained in a breast cancer cell line, suggesting that GOLPH3 may have a general role in glycolipid metabolism in multiple tumor types. Several features such as the organization, stoichiometry and composition of glycolipid glycosyltransferases complexes may modify their sorting properties, sub-Golgi localization, enzymatic activity and in consequence, the pattern of glycosylation at the plasma membrane. Here, we describe a new glycolipid glycosyltransferases complex between β3GalT-IV and ST3Gal-II. Furthermore, we show that GOLPH3 plays a crucial role in the formation of this glycolipid glycosyltransferases complex and by doing so it influences the glycolipids profile that human glioblastoma and breast cancer cells express at the cell surface. Finally, we observed that GOLPH3 also modified the levels of ST8Sia-I in both Golgi complex and plasma membrane from CHO-K1 cells stably expressing ST8Sia-I. In addition, the levels of GD3 ganglioside were also affected by the overexpression of GOLPH3. It is worth mentioning that ST8Sia-I (GD3 synthase) is a key enzyme that regulates the biosynthesis of b- and c-series gangliosides and therefore exerts a significant effect on cancers. In conclusion, this novel level of regulation of glycan synthesis opens up new questions to explore the molecular characterization of other complexes, where the oncoprotein GOLPH3 may also operate and participate in glycosylation pathways as well.

Session 4 – Neurodisease

Neural Heparan sulfates in tissue homeostasis and disease. The case of Alzheimer's disease.

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Proteoglycans are complex macromolecules formed of glycosaminoglycan chains covalently linked to core proteins through a linker tetrasaccharide common to heparan sulfate proteoglycans (HSPG) and chondroitin sulfate proteoglycans (CSPG). Biosynthesis of a single proteoglycan requires the expression of dozens of genes, which together create a large structural and functional diversity reflected by the numerous diseases or syndromes associated to their genetic variability. Among proteoglycans, HSPG are the most structurally and functionally complex. To decrease this complexity, we retrieved and linked information on pathogenic variants, polymorphism, expression, and literature databases on the dozens genes involved in the biosynthesis of HSPG core proteins, heparan sulfate (HS) chains, and their linker tetrasaccharide. This retrieval afforded the identification of 'Ubiquitous HS sequences' (uHS) involved in the maintain of tissue homeostasis and of 'Tissue specialized HS sequences' (sHS) involved in specific tissue vulnerability to disease. The talk will focus in the involvement of neural sHS in the mechanisms leading to the abnormal phosphorylation and aggregation of tau protein (tau pathology) in Alzheimer's disease (AD), the main form of dementia in the world. We will show the specific structural features of the sHS that accumulate in the AD brain and how these neural sHS contribute to the cellular mechanisms leading to the oligomerization and aggregation of tau in a cell autonomous manner. Our data support the central implication of sHS in the cellular mechanism leading to tau protein oligomerization and aggregation in AD and open to new therapeutic strategies.

Heparin-binding properties and putative chaperone activity of GAPDH in the extracellular space.

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Glyceraldehyde - 3 - phosphate dehydrogenase (GAPDH) is considered a multifunctional protein due to its ability to perform different functions in addition to its glycolytic role. This functional plasticity is achieved through conformational changes that expose new interaction surfaces with other proteins. In the extracellular space, glycosaminoglycans induce the conversion of GAPDH into multimeric species that can act as chaperones sequestering α-synuclein aggregates tempering their toxic effect. Nevertheless, changes in glycosaminoglycans associated to aging could affect this proteostatic mechanism. Here, the binding of GAPDH to glycosaminoglycans is characterized at a molecular level for the first time. Using X-ray crystallography, we identified the

heparin binding pocket located between the P and R subunits along the Q pseudo symmetry axis. Through fluorescence titration studies the apparent binding constant was estimated to be around 9 ± 4 μM−1, consistent with a protein of medium affinity for heparin, with a stoichiometry of approximately 3 ligands per GAPDH tetramer. In silico studies revealed 6-O and 2-O sulfation of glucuronic and iduronic acid respectively as the major contributors for the binding energy on the glycosaminoglycan structure. While the minimal unit for binding was determined to be a heparan sulphate trisaccharide, the minimal length to induce GAPDH conversion to multimeric species was established to be fifteen saccharide units. Site-directed mutagenesis allowed the identification of the amyloidogenic region organized around Trp-313 of GAPDH. These results open a venue to explore the development of new therapeutic strategies to slow down the progression of neurodegenerative diseases such as Parkinson's disease. The determination of pharmacophores in glycosaminoglycans establishes the necessary basis for the design of molecules capable of binding to GAPDH and enhancing its neuroprotective effect in the extracellular space.

Galectins in the microglial response in a model of Alzheimer's disease.

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Neuroinflammation has been proposed as a common factor and one of the main inducers of neuronal degeneration in Alzheimer’s disease (AD). Galectins are a group of β-galactoside-binding lectins that play an important role in the regulation of the microglial response and neuroinflammation. Up to 15 members of the galectin’s family have been identified; however, the expression of Gal-1 and Gal-3 has been considered a key factor in neuronal regeneration and modulation of the inflammatory response. Moreover, the expression of Gal-9 and receptor TIM3 is considered a key factor in the modulation of the microglial response. Recent evidence has shown that the administration of amyloid-β 25–35 (Aβ25–35) peptide into the hippocampus of rats increases the inflammatory response that is associated with memory impairment and neurodegeneration. Galectins could participate in the modulation of the neuroinflammation induced by the Aβ25–35 peptide. We evaluate the expression changes of Gal-1, Gal-3, Gal-9 and TIM3 in the neuroinflammation induced by the administration of Aβ25–35 peptide into the hippocampus or lateral ventricles and examine cognitive deficit. After the administration of Aβ25–35 peptide, animals were tested for learning and spatial memory in the Morris water maze (MWM) and novel object recognition test (NOR). Behavioral performance showed that Aβ25–35 peptide did not affect spatial learning but did impair memory in the MWW and NOR. On the day 32, hippocampus was examined for astrocytes (GFAP), microglia (Iba1), Gal-1, Gal-3, Gal-9 and TIM3 via immunohistochemical analysis, western blot, and the cytokines IL-1β, TNF-α, IFN-γ by ELISA. Results in this study showed a significant increase in the

expression of Gal-3 in the microglia and astrocytes, while Gal-1 did not increase in the dorsal hippocampus. The expression of Gal-9 and TIM3 that was detected predominantly in the cells of microglia respect to the control group. The expression of galectins is associated with increased cytokines in the hippocampal formation of Aβ25–35 peptide treated rats. Moreover, the increase in Gal-9 and TIM3 could be attenuate injuries in the peak phase of the inflammatory response, in neurodegenerative process induced by Aβ25-35 peptide. These findings suggest that Gal-3 could participate in the inflammation induced by the administration of Aβ25–35 peptide and could be involved in the neurodegeneration progress and memory impairment. Due to its anti-inflammatory effects, Gal-1 is proposed as a molecule with therapeutic potential, whereas the inhibition of Gal-3 could contribute to reducing the neuroinflammatory. Furthermore, the potential use of Gal-9 and TIM3 as therapeutic targets in the modulation of microglial cells promotes the remodeling of damaged tissues in the central nervous system.

Heparan sulfate (HS) mimetics; novel therapeutics for CNS repair.

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Demyelination and axonal pathology in the central nervous system (CNS) is associated with numerous neurological disorders such as spinal cord injury (SCI) and multiple sclerosis (MS). Any repair strategies for CNS damage must have a multifactorial approach including promotion of axonal outgrowth, and remyelination. One such candidate is heparan sulphate (HS) mimetics which are glycomolecules of repeating disaccharide units synthesised from heparin (mHeps) to express varying levels of sulphation and lack coagulation activity. They share structural similarities to cellular HS which modulate a huge range of cellular functions. They are thought to regulate a variety of cell signalling by both sequestering ligands and acting as a cofactor in the formation of ligand-receptor complexes. We have demonstrated that low sulphated mimetics (LS-mHeps) enhance neurite outgrowth and myelination in vitro by sequestering molecules that inhibit myelination. For these compounds to translate effectively to the clinical we have tested small MW versions (LS-mHep7L) that are thought to have enhanced bioactivities. We showed as well as promoting neurite outgrowth and myelination in vitro they can also enhance repair in spinal cord slice cultures after lysolecithin induced demyelination. Moreover, treating animals in the experimental autoimmune encephalomyelitis (EAE) model with LS-mHep7L we observed improved animal outcomes, including restoration of pre-disease animal weight. This is thought to occur via the prevention of inflammation leading to axonal preservation and remyelination. In summary, data from provided from ex vivo and in vivo work supports the future clinical translation of these next generation heparin mimetics as a novel treatment for CNS diseases.

TGF-ß1 regulates 3-O-sulfated heparan sulfate biosynthesis in Alzheimer's disease related tau pathology.

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Background. Aging is the main risk factor for Alzheimer's disease (AD) characterized by a cognitive decline related to a synaptic disfunction (Chen et al., 2019). In AD brains, tau abnormal phosphorylation coexists with heparan sulfates (HS), suggesting that HS could be involved in the formation of neurofibrillary tangles (NFT) (Goedert et al., 1996). Accordingly, in vitro, the aggregation of tau is not possible in the absence of heparin, a highly sulfated HS carrying 3-*O*-sulfation (3S-HS). 3S-HS are the product of HS 3-*O* Sulfotransferase (HS3ST) enzymes (Sepulveda-Diaz et al., 2015). In AD, Heparan Sulfate-Glucosamine 3-Sulfotransferase 2 (HS3ST2) expression is higher in tau pathology vulnerable brain regions, such as the hippocampus and cortex (Huynh et al., 2019). Previously, we showed in a cellular model that HS3ST2 induces the cell autonomous aggregation of tau (Huynh et al., 2022). However, the mechanism leading to the increased expression of HS3ST2, and therefore increase in 3S-HS synthesis in AD, are unknown. Additionally, the effect of reducing the levels of HS3ST2 in tau oligomerization and aggregation in neurons is unknown. In brain of aged individuals, transforming growth factor TGFB1 is upregulated, modulating gene expression (Doyle et al., 2010). SMAD4 transcription factor is activated upon the dimerization of TGFB receptor targeting specific genes (Martin-Malpartida et al., 2017). SMAD4 contains genome wide recognition motifs that are present in the Hs3st2 promoter, suggesting a possible regulation.
Results. Here, we show that Hs3st2 expression is under regulation of Tgfb1 and that blocking pSmad2/3 reduces its levels. Accordingly, Hs3st2 levels were increased by increasing pSmad2/3. The importance of Tgfb1 coregulation with Hs3st2 was assessed by blocking the expression of Hs3st2, leading to a decrease in tau oligomerization and aggregation. Finally, we found that the reduction of 3S-HS enhances synaptic density, and presynaptic/postsynaptic connectivity.
Conclusion. Overall, we depicted the regulation of Hs3st2, and demonstrated its involvement in synaptic connectivity and tau aggregation, confirming the importance of 3S-HS in the physiopathology of AD.

Emerging pathways to neurodegeneration in lysosomal storage disorders.

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Mucopolysaccharidosis (MPSs) are a family of inborn errors of metabolism due to a deficiency of lysosomal enzymes necessary for the degradation of glycosaminoglycans (GAGs), generally referred as lyososomal storage disorders. This leads to primary storage of undegraded GAG fragments in the lysosome. Defective metabolism of the GAG heparan sulfate (HS) is necessary and sufficient for the onset of neurodegeneration and related dementia in children, as in MPS-IIIA, which is caused by mutations of the gene encoding the lysosomal enzyme N-sulfoglucosamine sulfohydrolase (SGSH). The mechanism responsible for neurodegeneration is unknown. Preclinical studies identified neuronal secondary storage of insoluble fibrils of α-synuclein (α-syn), amyloid β (Aβ), tau, and prion protein associated with neurodegeneration in MPS-IIIA. Similar findings were reported for other neurodegenerative MPSs (MPS-I, MPS-II, etc), all characterized by mutations in one of the genes involved in the catabolic pathway of HS. These findings have supported a lysosome-centered view of the pathology, which postulates that dementia and neurodegeneration in MPSs are due to the autophagy-lysosomal dysfunction, consequent to the primary and secondary storages. However, this hypothesis does not sufficiently account for how and why the neuronal accumulation of other GAGs, such as dermatan sulphate (DS), in other forms of MPSs (such as MPS-VI) does not lead to neurodegeneration and dementia. This poses the unexplored issue of the HS-specific pathological role in MPSs. HS is a structural component of the extracellular matrix and is essential for proper growth factor signaling; additionally, depending on the length and the pattern of sulfation, HS has been shown to trigger intracellular aggregation of proteins favoring their misfolding into insoluble fibrils. We will present published and unpublished evidence showing how altered structure and functions of HS in MPS-IIIA in vitro and in vivo models is associated with the many histopathological and behavioral dysfunctions characterizing MPS-IIIA, before the onset of lysosomal dysfunction. These findings provide completely novel insights into why defective degradation of HS, but not other GAGs that accumulate in brain lysosomes, is necessary and sufficient to lead to neurodegeneration in MPS.
This project has been supported by Cure Sanfilippo, Sanfilippo children Foundation and Safilippo fighters Grant to EDL and DPG.

An extracellular matrix therapy to optimize mesenchymal stem cell-based therapy for brain protection and repair after cerebral ischemia.

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Stroke is a devastating neurological disease worldwide. To date, thrombolysis and thrombectomy are the only treatments that can be applied to a

limited number of patients (~15%) due to a short therapeutic window and there is no therapeutic alternative for the remaining patients. Stroke provokes a rapid neuronal death and the destruction of the extracellular matrix (ECM), associated to the degradation of the glycosaminoglycans which are mainly composed of heparan sulfates (HS). OTR4132, a HS mimetic, is a synthetic polysaccharide that bind matrix proteins and growth factors and thus increases their bioavailability which ameliorates the cellular environment and promotes tissue repair. In addition, following stroke, numerous preclinical studies showed that mesenchymal stem cells (MSC) are able to protect the brain tissue and enhance recovery, however their actions are limited by the hostile environment in the damaged tissue.
Here, we demonstrated the ability of OTR4132 not only to confer a long-lasting neuroprotection and reduced functional deficits after ischemic stroke but also to potentiate the beneficial effects of MSC in rats. Moreover, RT-PCR and immunohistochemistry data from ischemic brains showed that OTR4132 enhances cellular repair responses such as neurogenesis and angiogenesis and protects/regenerates the ECM which can facilitate migration and/or survival of MSC to the ischemic hemisphere. These effects may involve an increase in the MSC migration/survival to the injured site and/or an improvement in secretion of these cells.
Overall, our work underlines that OTR4132 alone as well as in association with MSC, is a promising ECM-based therapeutic strategy to enhance brain protection/repair and functional recovery after an ischemic stroke.

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FLASH PRESENTATION

CHO.K1 vs. HEK-293 cell platforms to produce novel neurotherapeutic candidates.

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Neurological disorders represent a group of pathologies characterized mainly by the loss of neurons in different areas of the brain, which defines the characteristic symptoms of each affection. There are some medicines currently approved for neurodegenerative disorders treatment that help with the associated symptoms without improving the patient´s condition. In an effort to develop new treatments, erythropoietin (EPO) has been proposed as a neurotherapeutic candidate.
EPO is a 165 amino acids-consisting glycoprotein that bears three N-glycosylation sites and one O-glycosylation site. EPO is a biotherapeutic widely used for treating anemia since it is the main regulator of blood cell production. However, studies have shown that EPO exhibits neuroprotective, antiapoptotic, antioxidative and neuroplastic properties

in the different tissues including the central nervous system.
With the aim of blocking the erythropoietic activity (EA) but preserving its neurobiological action (NA), two hyperglycosylated human erythropoietin (hEPO) analogs were previously produced in our lab as neurotherapeutic candidates through glycoengeniering by hyperglycosylation. Thus, Mut 45_47 and Mut 104 were produced by CHO.K1 cells, affinity-purified and characterized, evidencing the incorporation of an extra N-glycan chain, the lack of their EA and the preservation of their NA.
Considering that less sialylated hEPO is produced by the brain and that human cell lines produce glycoproteins with simpler glycosidic structures, lower sialic acid content and higher receptor binding, HEK-293 was chosen to produce EPO muteins that could resemble the properties of brain-produced hEPO.
In this context, HEK-293 cells were transduced with mutein-coding lentiviral particles. The HEK-293-produced proteins were purified by immunoaffinity chromatography showing yields between 43% and 60% and purities higher than 90% in a single purification step.
The partial N-deglycosylation procedure demonstrated a maximum expected occupancy degree of four N-glycosylation sites both in CHO.K1 and in HEK-293-produced variants. Besides, HEK-293 variants showed lower molecular mass than those produced by CHO.K1 cells. This difference could be due to less complex glycan structures as a consequence of a lower antennarity and/or a lesser sialic acid content, resembling the features of brain-produced hEPO. The increase in glycosidic content of CHO.K1 and HEK-293-derived muteins practically did not affect the temperature of thermal denaturation compared to hEPO confirming that tertiary structure was preserved after glycoengineering.
In terms of biological activity, HEK-293-produced proteins did not show *in vitro* EA and preserved NA as the corresponding analogs produced in CHO.K1 cells.
In summary, in this work we have produced, purified and characterized two hEPO variants which exhibited promising properties for treatment of neurological disorders.

Session 5 – Biotechnology and Chemical Biology

Protein glycoengineering: towards novel biotherapeutics.

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Protein-based biotherapeutics have transformed the treatment of several disorders due to their high specificity and affinity towards its clinical target. Unfortunately, most proteins do not perform as ideal drugs since their efficacy is compromised by numerous intrinsic limitations related to their pharmacokinetic and/or pharmacodynamic properties. Thus, many approaches have been developed aiming to improve the residence time of proteins in circulation to reach appropriate therapeutic doses, as well as to improve other physicochemical and pharmacological properties.
Most eukaryotic cells-derived proteins undergo covalent modifications either during or after their synthesis. This creates the concept of co-translational

and post-translational modifications (PTM). N- and O-glycosylation are the most abundant and complex PTM that proteins can undergo, affecting diverse biological properties, including solubility, protease and thermal stability, antigenicity, immunogenicity, bioactivity, and pharmacokinetics. Thus, glycosylation represents one of the most significant attributes of many therapeutic proteins, defining their potency and effectiveness. Also, both size and charge of proteins are completely modified by the presence of glycans, so that manipulation of this PTM represents a valuable tool to alter the pharmacokinetics and the pharmacodynamics of biotherapeutics.
Herein, I summarize the work carried out in our lab using two iconic glycoproteins that were modified employing glycoengineering by hyperglycosylation, i.e., a procedure to add new glycosyl moieties by introducing potential glycosylation sites. In this sense, potential biobetters and innovators were produced using animal cell cultures.
One of the glycoproteins, interferon-α2b -an antiproliferative, immunomodulatory and antiviral cytokine- was N-glycomodified or O-glycomodified to produce hyper N-glycosylated or hyper O-glycosylated versions of it with improved pharmacokinetic properties. The second glycoprotein was erythropoietin that was N-glycoengineerized to block its erythropoietic function while preserving its neurobiological action.
In particular the N-glycoengineerized erythropoietin led to the foundation of a start-up company and phases of patent nationalization as previous steps and essential milestones to move towards proof of concept tests, as well as preclinical and clinical assays to set up a novel and effective biotherapeutic.

Contributors: María de los Milagros Bürgi, Natalia Ceaglio, Matías Depetris, Marina Etcheverrigaray, Agustina Gugliotta, Francisco Iturraspe, Ricardo Kratje, María J. Leopold, Valentina Wandel Petersen.

Combined effect of ammonia stress and cell lineage on CHO cell derived VRC01 monoclonal antibody glycosylation.

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Key Words: CHO, ammonia, cell line age, glycan analysis

Glycosylation is a critical post-translational modification of proteins in mammalian cells. Glycosylation of monoclonal antibodies (mAbs) is of particular interest due to the effect it has on the effector function, structure and stability of the mAb, thus making it an important product quality attribute. To achieve a desired therapeutic effect, it is necessary to produce an antibody with a specific glycosylation profile. To produce this antibody, a better understanding of how changes to culturing conditions impact the glycoforms of mAbs is required. Ammonia concentrations typically increase during mammalian cell cultures, mainly due to glutamine and other amino acid consumption. In this work, we detect significant changes to glycosylation following ammonia stress of CHO-produced VRC01, a broadly neutralizing antibody undergoing clinical trials to treat HIV. Ambr250 bioreactors were run in parallel with varying concentrations of ammonia stress on CHO cells of two cell line ages. To determine the glycan profiles, the culture supernatant was collected over the course of the run and VRC01 was purified using gravity flow

chromatography with protein A resin. Glycans were fluorescently labeled and released from the mAb and eluted from a BEH Amide column using a Waters AQUITY H-class UPLC. We found that increased ammonia concentrations led to a decrease in total and terminal galactosylation, sialylation, and fucosylation of the associated glycans. While age did not have an effect on antibody galactosylation and sialylation under no ammonia stress, reduced galactosylation and sialylation was more severe for the more aged cell line when ammonia stress was present. This observed reduction in sialylation of glycoproteins following ammonia stress is consistent with previously published results [1-2]. Antibody fucosylation was found to be reduced in the more aged cell line in all cases, but ammonia stress did not exacerbate this effect.

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Raman spectroscopy-based monitoring of amino acids and antibody N-glycosylation in high cell density perfusion culture.

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Raman probe spectroscopy provides a process analytical technology (PAT) tool for monitoring and control of culture parameters in bioprocesses and has attracted interest in industry. An advantage is that the sensor is an immersed and autoclavable probe, providing on-line information about one or several culture parameters. Its usage however requires to decipher this information from a complex spectrum. This is achieved by developing predictive models.

In the present study, Raman-based PAT was developed for a process of high cell density steady-state perfusion culture of Chinese Hamster Ovary cells producing a recombinant antibody. A feature of steady-state cultures is that their behavior is rather stable. The modelling of the Raman spectrum is therefore more challenging, due to the absence of variations of the culture parameters. For this reason, the variations were created by generating a dynamic culture environment favorable for the model calibration thanks to different steady-states, obtained by varying the cell concentration up to 100 E6 cells/mL as well as the perfusion rate during the process development. It was also observed that the culture performances were similar for all the studied cell densities, but that the cell specific perfusion rate (CSPR) strongly influenced the cell metabolism, the productivity of the recombinant antibody and its N-glycosylation. Partial least squares models were obtained for several parameters, such as the cell density and the concentrations of lactate, ammonium and amino acids. Furthermore, a correlation was observed between the concentrations of the amino acids and the antibody N-glycosylation, which was then modeled to predict this glycosylation pattern, providing a real

time tool. The models were calibrated on several runs and then validated with new data showing prediction accuracy.
The present approach integrating a Raman probe can provide a valuable PAT tool for monitoring and control steady-state perfusion production processes.

Next-gen glycoengineering: Combining cellular and metabolic engineering to fine-tune mAb β1,4-galactosylation.

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N-linked galactosylation is a major source of heterogeneity in commercial monoclonal antibody (mAb) products [1]. Despite the clear need to tightly control mAb galactosylation, strategies to do so remain limited: standard cell engineering strategies are not amenable for real-time control applications, advanced cell engineering strategies require multiple time-consuming genetic engineering events [2, 3], and metabolic glycoengineering strategies achieve only a narrow range of control at the expense of cell growth and product yield [4, 5]. Here, we present a novel approach that enables real-time galactosylation control across a broad range by feeding a decoy substrate, 2-deoxy-2-fluoro-d-galactose (2FG), to CHO cells that have been engineered to produce hypergalactosylated mAb product.
CHO-DP12 cells producing up to 98% galactosylated mAb were cultured in fed-batch mode, where 2FG was fed to achieve concentrations ranging from 0.1mM to 2mM. LC-MS glycoprofiling [6] of purified mAb revealed that our glycoengineering strategy achieved dose-dependent control of product galactosylation ranging from 45% to 93%. No detrimental effect of 2FG feeding on cell growth and mAb titre was observed with up to 2mM of 2FG. With increasing 2FG concentrations, an accumulation of undesired aglycosylated mAb product was observed. Upon further analysis, the lowest achieved level of mAb galactosylation (45% galactosylated species) was observed to occur because half of all mAb (at 93% galactosylation) is produced before the first 2FG feed and not due to limited 2FG activity. To confirm the robustness of our glycoengineering strategy, it was tested in a second hypergalactosylating mAb-producing cell line, CHO-VRC01. A 2FG range between 10pg and 80pg was fed on days 3, 4 and 5 aiming to maintain a stable amount of inhibitor from the exponential phase onwards, with no apparent effect observed on either cell proliferation or mAb titre. With the above 2FG feeding strategy, a dose-dependent product galactosylation control range of 57% to 94% was achieved. Importantly, for the revised 2FG feeding strategy, only minimal levels of product aglycosylation were observed.
Work is ongoing to expand the range of galactosylation controllability and to test the overall strategy in additional cell lines. With this work, we aim to establish our next-generation glycoengineering approach as a tool for real-time control of mAb glycosylation that will, ultimately, contribute to quality assurance during biopharmaceutical manufacturing.

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Chemical Precision Tools to Dissect Protein Glycosylation.

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Alterations in glycoprotein expression and composition are an undisputed corollary of cancer development. Consequently, some of the most faithful tumor biomarkers are heavily glycosylated. Understanding cancer-related changes of the glycoproteome is paramount but hampered by limitations in cellular model systems: Technological advances in mass spectrometry have allowed profiling of glycoproteomes but are often restricted to isolated cells that do not adequately reflect the interaction between tumor and microenvironment. Co-culture systems in vitro or in vivo better reflect the physiological environment but the glycoproteomes of cells from the same host organism cannot be meaningfully discerned to distinguish tumor from associated cells.
Here, we report the development of chemical “Precision Tools” that allow for Bio-Orthogonal Cell-specific Tagging of Glycoproteins (BOCTAG). We equip cells with an artificial metabolic pathway to biosynthesize chemically tagged UDP-GalNAc analogues. Engineered glycosyltransferases accommodate these chemical tags, allowing to selectively study the glycoproteome of BOCTAG-transfected cells in the presence of bystander cells. We extensively validate BOCTAG as a strategy for cell-specific imaging in co-culture and to selectively annotate cell-specific glycosylation sites by mass spectrometry. BOCTAG serves to visualize and profile the cancer-specific glycoproteome in co-culture in vivo and in vitro without cell sorting and in secretome, unraveling the importance of glycosylation as a modulator of cellular function.
Recently, we have applied the principles of BOCTAG and other chemical tools to unveil O-GalNAc glycosylation as a modulator of the proteolytic maturation of SARS-CoV2 spike, majorly influencing the mutational trajectory of variants of concern. Our work highlights the outstanding relevance of chemistry for glycobiology.

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A Novel System for Glycosylation Engineering by Natural and Artificial miRNAs.

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N-Glycosylation is a critical quality attribute of many therapeutic proteins and a common post translational modification. The expression of consistent and human-like glycosylation pattern is still a challenge for bioproduction. Here we present a novel system for glycosylation engineering using natural and artificial miRNAs. This technology enables glycoengineering of host cell lines to modulate and fine-tune protein glycosylation during biopharmaceutical production.

FLASH PRESENTATIONS

Albumin-bovine submaxillary core shell nanoparticles as a novel submaxillary antibiotic-delivery system to target *Campylobacter jejuni.*

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Infectious gastroenteritis caused by *Campylobacter jejuni* is the most common worldwide. *Campylobacter* infections are generally mild, but *can be fatal* among very young children*,* elderly*,* and immunosuppressed individuals and in some cases leads to complications for the rest of people. In addition, antibiotic-resistant-*Campylobacter* strains are highly prevalent in many countries. Selective rifaximin targeting in functionalized nanoparticles can be an alternative for treating local infection. *C. jejuni* has adhesins that recognize sialylated structures to bind to enterocytes. In this study, rifaximin-loaded core-shell sialylated nanoparticles (NPs) were synthesized and characterized as active vectors targeting *C. jejuni*. Bovine submaxillary mucin (Muc-Sial) was used as a source of sialic acid glycoconjugates. The core nanoparticle was synthetized with bovine serum albumin (BSA) loaded with rifaximin while the Muc-Sial shell was crosslinked with glutaraldehyde, forming BSA- Muc-Sial NPs. The FTIR-ATR spectra confirmed the NPs functionalization. The encapsulation efficiency of rifaximin was 24 % and the minimum inhibitory concentration for *C. jejuni* was 1 mg/mL. BSA-Muc-Sial nanoparticles were recognized by *Maackia amurensis* sialic acid specific lectin. NPs also were recognized by C. *jejuni* adhesins in a temperature-dependent manner, indicating the differential expression of adhesins in *C. jejuni*. This study indicates that under *in vitro* conditions the synthesized sialylated nanoparticles loaded with rifacimin can be successfully delivery to *C. jejuni*.

Impact of variants on human α-L-Iduronidase glycosylations displacements.

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Human α-L-Iduronidase (IDUA) is a glycosidase involved in the sequential degradation of glycosaminglycans (GAGs) heparan and dermatan sulfate. IDUA possess 6 N-glycosilations, one of which has been reported to be involved in substrate binding and as catalytic module. The expression of variants in IDUA leads to decreased enzyme activity resulting in the accumulation of undegraded heparan and dermatan sulfate, causing mucopolysaccharidosis type I (MPS I). To date, the decrease in enzyme activity of IDUA variants has been associated with structural alterations; however, the impact of variants located in different domains on the molecular dynamics of glycosilations had been poorly evaluated. A 50 ns MD simulation was performed, and representative structures were evaluated at different times of the MD for WT IDUA and for P533R, S633L and S633W variants, located on the β-sandwich and Ig-like domains, respectively. Each variant is associated with a different MPS I phenotype. Molecular dockings carried out between a tetrasaccharide derived from dermatan sulfate and the representative structures of MD, showed that the region conformed between N-glycan of Asn372 and Asn415, may be a binding region for the GAGs. A structural analysis showed that the molecular dynamics of the glycosilations of the evaluated variants differ from those observed for the WT enzyme. Throughout the MD, the N-glycan linked to Asn372 presented structural changes that could allow the opening and closing of the catalytic pocket. However, unlike the S633L and P533R variants, in S633W variant the Asn372-linked N-glycan hardly reaches coordinates close to those observed in the WT enzyme along the MD to close the catalytic pocket. Displacements of ~2 Å in the substrate-binding mannose were observed in the N-glycan attached to Asn372 for the minimized structure, and of ~4 Å at 50ns. Furthermore, for Asn415 at 0ns, a shift of ~3.8 Å led to a significant displacement in the coordinates of the N-glycan-linked. In contrast, Asn110-linked N-glycan exhibited a lower impact given by the variants, on its molecular dynamics. IDUA N372-glycan has been shown to be essential for enzyme function, therefore, an evaluation of the molecular dynamics of the glycans of IDUA variants might led to a better understanding of the pathology. To date, the rest of N-glycans have not been associated with a significant decrease in IDUA catalytic activity, however, due to the proximity of the Asn415-linked N-glycan to the GAG-binding region, these glycans might participate in substrate binding. Alterations in the molecular dynamics of glycosilations, along with other structural changes, could explain the decrease in enzyme catalytic activity and, according to our results, might also be associated with a specific phenotype, at least for the variants associated with the severe phenotype of MPS I.

Identification and characterization of deaminoneuraminic acid (Kdn) - specific aldolase (Kdn-aldolase) from *Sphingobacterium* sp.

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Sialic acids (Sia) are a group of acidic sugars with a nine-carbon backbone. Sias are divided into three fundamental species; *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (Kdn). Sias are presented on the termini of glycans and involved in various biological phenomena such as fertilization, development, infection, and tumorigenesis. Neu5Ac metabolism has already been clarified in bacteria. Terminal Neu5Ac residues are hydrolyzed by sialidase. The released Neu5Ac is cleaved into *N*-acetylmannosamine (ManNAc) and pyruvate by sialate-pyruvate lyase (SPL) or Neu5Ac aldolase (NanA). In contrast, Kdn metabolism is not well understood except that Kdn-glycans are resistant to most bacterial sialidases. We previously found a Kdn-specific sialidase (KDNase) from *Sphingobacterium* sp existed in soils. We thus thought the bacteria also had Kdn-specific aldolases for Kdn catabolism. In this study, we explore the Kdn-specific aldolase (Kdn-aldolase) from *Sphingobacterium* sp. Whole-genome sequencing revealed that the bacteria had four candidate genes: ORF1728, ORF2211, ORF4424 and ORF4464. Then, we investigated the enzymatic properties of the candidates. The enzymatic activity assay revealed that the ORF2211 was Kdn-aldolase. The Kdn-aldolase had the optimal pH and temperature at 7.0-8.0 and 50 °C, respectively. In addition, the enzyme had Kdn synthetic activity. Amino acid alignment and homology modeling suggested Asn50 in the Kdn-aldolase was important in Kdn recognition. The specificity was changed by site-directed mutagenesis of the Asn50 residue, suggesting that the Asn50 is important for Kdn degradation. The finding of the Kdn-aldolase indicates that the Kdn-specific metabolism is important in nature like Neu5Ac metabolism.

Session 6 – Genetics

Congenital Disorders of Glycosylation - diagnostic challenges solved by glycoproteomics.

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Congenital Disorders of Glycosylation (CDG) form a fast growing group of genetic defects that are characterized by abnormal glycosylation of proteins and lipids. At the start of the CDG era, most defects were identified in protein N-glycosylation, for which a very simple screening test was available; transferrin isofocusing. Due to the advent of next-generation sequencing, more than 140 different genetic diseases are currently known for a variety of glycosylation pathways, such as *O*-mannosylation, glycosaminoglycan synthesis, lipid glycosylation, and N-glycosylation. This has resulted in a need for better biomarkers for diagnostics with increased specificity and sensitivity, and for biomarkers that can predict disease progression and therapy response.
This presentation will mainly focus on CDGs due to defects in protein N-glycosylation. Screening for defects in protein N-glycosylation is occurring broadly via analysis of plasma transferrin by isofocusing, HPLC or capillary electrophoresis. Increased specificity and sensitivity is obtained by

high-resolution mass spectrometry of intact plasma transferrin, which allows for screening and CDG subtyping in a single diagnostic test. For example, diagnostic profiles are obtained for MAN1B1-CDG and PGM1-CDG, and newly identified STX5-CDG. For some subtypes, dedicated diagnostic tests are required such as urine oligosaccharide profiling for NGLY1-CDG and MOGS-CDG, or analysis of sugar metabolites in patient cells for NANS-CDG.
More sophisticated glycomics and especially glycoproteomics techniques can be applied to study CDG defects that don't affect transferrin glycosylation, such as SLC35C1-CDG or MOGS-CDG. We have now implemented glycoproteomics in a clinical diagnostic setting, so that both CDG types can be diagnosed by glycosylation analysis of IgG. Although biomarkers for prognosis and therapy monitoring are not yet broadly applied in CDG, glycoproteomics technologies are very promising to obtain individualized insights in treatment response in CDG, as will be discussed for PGM1-CDG and SLC35C1-CDG. The initiation of CDG natural history studies in several parts of the world will prove to be instrumental to obtain well-characterized patient materials to identify biomarkers for prognosis, which will form the basis for monitoring the effect of future treatments.

TMEM260 is a protein-specific O-mannosyltransferase.

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Protein *O*-linked mannose (*O*-Man) glycosylation is an evolutionary conserved post-translational modification that fulfills key functional roles during embryonic development. Loss-of-function mutations in glycosyltransferases involved in initiation and assembly of *O*-Man glycans lead to developmental disorders, including muscular dystrophies and brain malformations. Recent advances in precise genome editing and glycoproteomics have revealed that initiation of protein *O*-Man glycosylation is controlled by the non-redundant GT-C enzymes POMT1/POMT2 and TMTC1-4, which are responsible for attachment of *O*-Man to alpha-dystroglycan (αDG) and the cadherin superfamily of adhesion molecules, respectively. However, the enzyme and biosynthetic pathway responsible for *O*-Man glycosylation of the third class of structurally related but functionally distinct transmembrane plexin receptors, hepatocyte growth factor receptor (HGFR) and macrophage-stimulating protein receptor (MST1R), has remained unknown.

To identify the putative glycosyltransferase responsible for *O*-Man glycosylation of plexins, HGFR and MST1R receptors, we performed a bioinformatics screen and mapped the *TMEM260* gene, encoding a previously uncharacterized multi-pass transmembrane protein, as a promising candidate. CRISPR/Cas9 knock-out (KO) of *TMEM260* in HEK293 cells, combined with quantitative *O*-glycoproteomics, demonstrated selective loss of O-Man glycosylation on plexins, HGFR and MST1R without affecting αDG or cadherin superfamily O-Man glycosylation. Biallelic mutations in *TMEM260* cause structural heart defects and renal anomalies (SHDRA) syndrome, and using cell-based models, we demonstrate that *TMEM260* disease variants have a markedly reduced ability to induce *O*-Man glycosylation on plexins, HGFR and MST1R receptors.

We further find that lack of *O*-Man glycosylation influences receptor maturation and causes abnormal growth in 3D cell models.

Our study uncovers a new biosynthetic pathway for mammalian *O*-Man glycosylation, with dedicated functions to a specific class of plasma membrane receptors, and identifies the SHDRA syndrome as a new type of congenital disorder of glycosylation.

Modeling glycosylation biosynthesis to understand glycogene and glycan structure relationships.

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We have been developing a Web-based tool called GlycoSim [1], which can predict the biosynthesis pathway of glycans, construct a mathematical model, and perform time-elapsed simulations of changes in concentration of enzymes and glycans. Using GlycoSim, we have simulated the biosynthesis of N-glycans in mouse ES cells obtained from experiments by Nairn et al. [2]. When performing simulation, reaction parameter values such as the rate coefficient (kf) of the enzyme reaction formula, the dissociation constant (Km) between the glycan and the enzyme, and the dissociation constant (Kmd) between the sugar nucleotide and the enzyme are required, but the majority of the values of these reaction parameters are unknown. Therefore, we aimed to determine the range of each reaction parameter value by estimating unknown parameter values. In this study, GlycoSim was used to construct a mathematical model of N-glycans, and COPASI [3] was used to perform parameter estimation 10 times using the Particle Swarm method. Using the best set of estimated parameters, simulation was performed, and the squared error between the experimental and simulated values was calculated. Among the variety of parameters estimated, the minimum squared error observed was 3.19E-14, which is extremely close to the experimental values. In order to further narrow down the range of the estimated reaction parameter values, the parameters were estimated several times by narrowing the estimation range with reference to the parameter set with the smallest squared error, and the range of the reaction parameters was determined. A Gaussian distribution of all the estimated values for each parameter could then be obtained. In the future, we plan to create a database of these data and make them accessible from the GlyCosmos Portal [4]. These parameters will then be available to anyone using GlycoSim and apply them to their own models and simulations. Through the foundational infrastructure developed through this GlycoSim project, we aim to aid glycobiologists in understanding glycan structure-function relationships.

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Toward Finding a Cure for NGLY1 Deficiency.

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The cytoplasmic peptide:N-glycanase (PNGase; NGLY1 in humans) is a deglycosylating enzyme widely conserved in eukaryotes [1]. This enzyme is involved in the degradation of misfolded *N*-glycosylated proteins destined for proteasomal degradation in the cytosol, a process called endoplasmic reticulum-associated degradation (ERAD). The discovery of NGLY1 deficiency, a human genetic disorder bearing mutations in the *NGLY1* gene, has led to rapid research progress on this protein [2]. We have been analyzing various *Ngly1*-KO animals (mice/rats/flies), and have identified genetic factor(s) greatly affecting the phenotypic consequences of these animals [3, 4]. It is particularly worth noting that additional KO of *Fbs2*, a glycan-recognizing E3 ubiquitin ligase subunit, can rescue the embryonic-lethal phenotype of *Ngly1*-KO mice, and the *Ngly1 Fbs2* double-KO mice exhibited no obvious defect on their motor functions [4]. These genes can thus be regarded as a promising drug target for NGLY1 deficiency. Moreover, recent evidence also suggested that AAV9-based gene therapy could be a viable therapeutic option for NGLY1 deficiency [5]. In this lecture, I will summarize the most recent progress of our efforts to find a cure for NGLY1 deficiency.

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Dysregulated Lysosomal Exocytosis Drives Protease-Mediated Cartilage Pathogenesis in Multiple Lysosomal Disorders.

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The classic view of the lysosome as a static recycling center has been replaced with one of a dynamic and mobile hub of metabolic regulation. This revised view raises new questions about how dysfunction of this organelle causes pathology in inherited lysosomal disorders. In prior studies using a zebrafish model for mucolipidosis II (MLII), our laboratory elucidated a pathogenic cascade in cartilage that

leads to the craniofacial defects in this model. We demonstrated that hypersecretion of a protease, cathepsin K, due to impaired M6P-dependent lysosomal targeting leads to its activation and sustained extracellular activity. This in turn disrupts the growth factor signaling pathways that drive normal chondrogenesis. In an effort to identify other mechanisms whereby the release of lysosomal proteases is relevant to pathogenesis, we developed zebrafish models for two other lysosomal diseases- sialidosis (neu1) and MPSIVA (galns), showing that lysosomal exocytosis is dramatically increased in the developing cartilage of lysosomal disease zebrafish models with distinct etiologies. Dysregulated exocytosis was linked to altered increased activity of multiple cathepsin proteases, and cathepsin- and TGFß-mediated pathogenesis in these models. Moreover, inhibition of cathepsin activity or direct blockade of exocytosis with small molecule modulators improve the cartilage phenotypes, reinforcing a connection between excessive extracellular protease activity and cartilage pathogenesis. This study highlights the pathogenic consequences in early cartilage development arising from uncontrolled release of lysosomal enzymes via exocytosis, and suggests that pharmacological enhancement of this process could be detrimental during tissue development.

Therapeutic targeting of POGLUT1 in a genetic liver disease.

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Paucity of intrahepatic bile ducts is caused by various etiologies and often leads to cholestatic liver disease. For example, in patients with Alagille syndrome (ALGS), which is a genetic developmental disorder primarily caused by mutations in the Notch pathway ligand *JAG1*, bile duct paucity often results in severe cholestasis and liver damage. However, no mechanism-based therapy exists to restore the biliary system in ALGS or other diseases associated with bile duct paucity. Based on previous genetic observations, we sought to investigate whether postnatal knockdown of the glycosyltransferase gene *Poglut1* (protein *O*-glucosyltransferase 1) can improve the ALGS liver phenotypes in several mouse models of this disease.

To this end, we first established an antisense oligonucleotide (ASO) which can efficiently knockdown *Poglut1 in vivo*. We then used this ASO to show that reducing *Poglut1* levels in postnatal livers of ALGS mouse models with moderate to profound biliary abnormalities can significantly improve bile duct development and biliary tree formation. Importantly, ASO injections prevent liver damage in these models without adverse effects. Furthermore, ASO-mediated *Poglut1* knockdown improves biliary tree formation in a different mouse model with no *Jag1* mutations. Previous work has shown that POGLUT1 adds *O*-linked glucose residues to epidermal growth factor-like repeat motifs with a specific consensus sequence, which are found in Notch pathway receptors and ligands as well as tens of other mammalian proteins. Using cell-based signaling assays, we find that reducing POGLUT1 levels or mutating POGLUT1 modification sites on JAG1 increase JAG1 protein level and JAG1-mediated signaling, suggesting a likely mechanism for the observed *in vivo* rescue. Our preclinical studies establish ASO-mediated *POGLUT1* knockdown as a potential therapeutic strategy to promote biliary development in ALGS and possibly other diseases associated with bile duct paucity.

GestaltMatcher: an AI that can recognize characteristic phenotypic patterns of many genetic disorders.

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Deep convolutional neural networks (DCNNs) can be trained to recognize and classify rare genetic disorders based on their characteristic facial features. GestaltMatcher is such an AI that learned to construct a clinical face phenotype space (CFPS) in which portrait images can be placed. Distances in between medical images in the CFPS can be used to quantify syndromic similarities. Cluster analysis in that space reveals close proximity of molecular pathway diseases and can, therefore, be used to support "lumping decisions". Furthermore, the pleiotropic genes causing multiple disorders can also result in distinct facial gestalt. Thus, cluster analysis can also provide evidence for "splitting decisions", that is indication for different pathomechanisms based on gestalt analysis.

FLASH PRESENTATIONS

The transmembrane domain of Endoplasmic Reticulum α-Glucosidase I influences the survival of a MOGS-CDG fission yeast model.

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Congenital Disorders of Glycosylation (CDG) are a group of rare genetic disorders that affect a person's ability to properly glycosylate proteins and lipids in their cells. These disorders can manifest in a wide range of symptoms, including developmental delays, neurological problems, and several organ dysfunctions. *N*-glycosylation of proteins is a highly conserved process

and one of the most significant post-translational modifications in eukaryotic organisms. During N-glycosylation the pre-assembled oligosaccharide $Glc_3Man_9GlcNAc_2$ (G3M9) is transferred by the oligosacharyltransferase complex to asparagine residues of proteins translocating into the ER. Then, ER-glucosidase I (GI) hydrolyzes the outermost glucose from G3M9, producing G2M9 glycoproteins enabling glucosidase II (GII) to convert G2M9 to G1M9, recognized by the ER Quality Control of glycoprotein folding (ERQC) mechanism. ERQC facilitates the folding of secreted and membrane proteins, and ensures that only proper folded ones continue to the Golgi. GI related defects lead to type IIb CDG (CDG-IIb or MOGS-CDG), a disease linked to an inactive GI. Our earlier findings indicated that the accumulation of G3M9 glycoproteins due to a GI deficiency in *Schizosaccharomyces pombe* is highly toxic to the cell, resulting in a sick phenotype in GI yeast mutants (ΔGI cells). Knocking alg10p alpha-1,2-glucosyltransferase, responsible for adding the outermost glucose residue during glycan biosynthesis, does partially –but not completely- suppress this adverse phenotype, indicating that bypassing GI requirement in glycoprotein processing is not enough to relieve the growth and morphological cell defects. On the other hand, most mutations observed in patients are not located in the catalytic pair residues of GI. These results suggest that the enzyme's catalytic domain may not be the only explanation for the defects observed in ΔGI cells, and that other domains of GI may also play a significant role. While GII is a soluble resident ER protein, GI is an ER membrane-bound one, comprising the globular catalytic domain within the ER, a transmembrane domain, and a cytosolic tail. In this work, we examined the phenotypic impact of expressing a catalytic-domain-only GI (cGI) in ΔGI mutant. We confirmed the proper expression and localization of the GI variant through fluorescence microscopy and western blotting. Its activity was assessed *in vitro* by using a fluorometric assay that detects hydrolyzed glucose from a synthetic oligosaccharide substrate specific of GI. We evaluated the cell viability and the growth rate of cGI expressing cells and measure cell lengths comparing with the expression of a full-length GI. Our findings demonstrate that cGI in ΔGI mutants is not able to rescue the sick phenotype observed in fission yeasts lacking the full-length GI, supporting the hypothesis that GI possesses an additional undiscovered role, potentially related to its ER membrane localization.

Highlighting Multidisciplinary Collaboration Between Clinicians And Basic Researchers Accompanying The Diagnosis And Treatment Of Congenital Disorders Of Glycosylation Towards Personalized Medicine In Latin-American Populations.

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The complexity of diagnosing inborn errors of metabolism in pediatric patients became a challenge because of the multidisciplinary approach necessary and the complex studies. Clinicians are faced with a heterogeneous group of diseases caused by mutations in human genes. Congenital disorders of glycosylation (CDGs) are one of these metabolic pathways that required complex studies of carbohydrate structures such as N- and O-glycoproteins, glycosaminoglycans, and other glycoconjugates (glycosphingolipid and glycosylphosphatidylinositol anchor among other). These structures are involved in multiple biological functions and glycan synthesis disruption results in multisystemic diseases with neurological involvement known as CDGs. These genetic human diseases include more than 150 affected genes, that remain widely under or miss-diagnosed in Latin America. The objective of this study is to highlight the complexity of the clinical and research collaboration necessary for a specific CDG diagnosis and treatment when available. The advances in the understanding of CDG physiopathology are required to detect novel biomarkers and new genes to support diagnosis and new treatments. This study includes patients from the Argentinean medical centers, presenting a multisystem phenotype with mild to severe psychomotor disability, hypotonia, seizures, failure to thrive, hormonal anomalies, coagulopathy, and cerebellar hypoplasia. Ethical permissions and informed consent were obtained. The complexity of the technical approach includes the study of gycoproteins (transferrin or Apo C-III) by isoelectric focusing, capillary electrophoresis or HPLC. Furthermore, the CDG diagnosis was confirmed by genetic testing including massive sequencing. Results: multidisciplinary studies resulted in the diagnosis of 15 patients more of these CDG are very rare worldwide (8 PMM2-CDG, 3 ALG2-CDG, 1 ALG13-CDG, 1 COG1-CDG, 1 ATP6AP2-CDG, 1 MAN1B1-CDG). Clinicians and researchers have been engaging the collaborations more effectively in the last years, encouraging families to get involved to participate. This advance represents an excellent opportunity to collaborate in a CDG Latam Consortium for professionals and families.

Session 7 – Glycoplants

Addressing the role of the nucleotide sugar transporter gene family in *Arabidopsis thaliana.*

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The plant cell wall, an intricate and dynamic structure, is a crucial determinant of cell shape, growth, and interaction with the environment. At its core lies a polysaccharide matrix, contributing to mechanical robustness, cellular adhesion, and responses to environmental cues. The synthesis of these essential cell wall polysaccharides is influenced by the availability of nucleotide sugar substrates, facilitated by nucleotide sugar transporters that shuttle these substrates into the Golgi apparatus.

This presentation addresses the role of UDP-Rhamnose/UDP-Galactose Transporters (URGTs) 1 to 6 in the biosynthesis of plant mucilage polysaccharides. The URGTs transport UDP-Rhamnose and UDP-Galactose into the Golgi apparatus, the organelle where cell wall polysaccharide biosynthesis takes place. Notably, mutations affecting URGTs 2, 4, and 6 exert discernible impacts on the composition and content of Rhamnogalacturonan I (RG-I) in plant mucilage. The consequences of these mutations extend beyond RG-I, influencing the composition of other monosaccharides, suggesting that URGTs are also involved in galactoglucomannan biosynthesis. The disruption in URGT function is met with an intriguing response: the up-regulation of gene expression pathways linked to cell wall biosynthesis in mucilage. This complex compensatory mechanism shows the plant's endeavor to compensate for the changes in cell wall integrity produced upon URGT mutations.

In conclusion, URGTs 2, 4, and 6 emerge as pivotal players in the construction of mucilage polysaccharides. The perturbation of these transporters yields insights into their specific functions and the broader regulatory network orchestrating plant responses to disruptions in cell wall biosynthesis.

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Next-Generation Plant Polysaccharides: From Parts Prospecting to Scalable Bioengineering of Cell Walls.

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As the most abundant source of renewable organic polymers on Earth, plant cell walls provide carbohydrates for food and feed, feedstocks for biofuels, and materials to build numerous products. Plants polysaccharides have heterogenous structures and are usually part of complex blends, so their underlying biosynthetic pathways been difficult to elucidate and modify. My talk will focus on recent advances in the production of hemicelluloses, which are composed predominantly of

beta-1,4-glucosyl linkages and are the dominant class of polysaccharide after cellulose. Although the number of plant genes known to influence cell wall β-glucans such as (gluco)mannan has gradually increased in the last two decades, conventional genetic approaches to alter their content and composition in plants yielded limited success so far. Therefore, many important questions remain regarding the molecular players that participate in biosynthesis, how they tailor polysaccharide structure, and where bottlenecks occur in living cells. The Designer Glycans laboratory addresses these gaps by applying synthetic biology approaches to gain insight into plant cell wall biosynthesis and modification.

To speed up biological discovery, my group is bioengineering yeast to gain mechanistic insight into the biochemical functions of plant β-1,4-linked (gluco)mannan synthases and their protein co-factors (Voiniciuc et al. 2019, *PNAS*). Recently, we assembled modular cellulose synthase-like enzymes to fine-tune the synthesis of plant mannans (Robert et al. 2021, *Biotechnol. Biofuels*) and have developed novel carbohydrate-binding probes for high-throughput detection of specific glycans. By building a biofoundry, we will start automating the design and build steps of cell wall bioengineering and thus drive a cultural shift towards larger-scale experiments. These strategies provide novel routes to develop SynBio circuits that make polysaccharides with new-to-nature functions, to break down recalcitrant biomass more efficiently, and/or to tailor crop traits towards a circular bioeconomy.

Structural and biochemical insight into a modular β-1,4-galactan synthase in plants.

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Rhamnogalacturonan I (RGI) is a structurally complex pectic polysaccharide with a backbone of alternating rhamnose and galacturonic acid residues substituted with arabinan and galactan side chains. Galactan synthase 1 (GalS1) transfers galactose and arabinose to either extend or cap

the β-1,4-galactan side chains of RGI, respectively. Here we report the structure of GalS1 from *Populus trichocarpa*, showing a modular protein consisting of an N-terminal domain that represents the founding member of a new family of carbohydrate-binding module, CBM95, and a C-terminal glycosyltransferase family 92 (GT92) catalytic domain that adopts a GT-A fold. GalS1 exists as a dimer in vitro, with stem domains interacting across the chains in a 'handshake' orientation that is essential for maintaining stability and activity. In addition to understanding the enzymatic mechanism of GalS1, we gained insight into the donor and acceptor substrate binding sites using deep evolutionary analysis, molecular simulations and biochemical studies. Combining all the results, a mechanism for GalS1 catalysis and a new model for pectic galactan side-chain addition are proposed.

Filing the gaps to solve the plant glycoprotein Extensin puzzle.

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Plant genomes encode a unique group of papain-type Cysteine EndoPeptidases (CysEPs) containing a KDEL endoplasmic reticulum (ER) retention signal (KDEL-CysEPs or CEPs). CEPs process the cell-wall scaffolding EXTENSIN proteins (EXTs), which regulate *de novo* cell wall formation and cell expansion. Since CEPs are able to cleave EXTs and EXT-related proteins, acting as cell wall-weakening agents, they may play a role in cell elongation. *Arabidopsis thaliana* genome encodes three CEPs (AtCPE1-AtCEP3). Here we report that the three Arabidopsis CEPs, *AtCEP1-AtCEP3,* are highly expressed in root-hair cell files. Single mutants have no evident abnormal root-hair phenotype, but *atcep1-3 atcep3-2* and *atcep1-3 atcep2-2* double mutants have longer root hairs (RHs) than wild type (Wt) plants, suggesting that expression of *AtCEPs* in root trichoblasts restrains polar elongation of the RH. We provide evidence that the transcription factor *NAC1* activates AtCEPs expression in roots to limit RH growth. Chromatin immunoprecipitation indicates that NAC1 binds the promoter of *AtCEP1, AtCEP2,* and to a lower extent to *AtCEP3* and may directly regulate their expression. Indeed, inducible *NAC1* overexpression increases *AtCEP1 and AtCEP2* transcript levels in roots and leads to reduced RH growth while the loss of function *nac1-2* mutation reduces *AtCEP1-AtCEP3* gene expression and enhances RH growth. Likewise, expression of a dominant chimeric NAC1-SRDX repressor construct leads to increased RH length. Finally, we show that RH cell walls in the *atcep1-1 atcep3-2* double mutant have reduced levels of EXT deposition, suggesting that the defects in RH elongation are linked to alterations in EXT processing and accumulation. Taken together, our results support the involvement of AtCEPs in controlling RH polar growth through EXT-processing and insolubilization at the cell wall.