(234) "High throughput analysis of intact N- and O-linked glycopeptides of the murine synaptosome"

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The first large scale study characterizing intact N- and O-linked glycopeptides will be presented. Lectin-affinity fractionation with wheat germ agglutinin was used for the enrichment of peptides bearing intracellular O-GlcNAc, but also those featuring ER/Golgi-derived N- and O-linked carbohydrate structures. Glycopeptide fractions were further fractionated off-line by high pH reversed phase chromatography and then subjected to LC/MS analysis on an Orbitrap Velos mass spectrometer. Precursor ions were measured by high accuracy in the Orbitrap, while high sensitivity electron-transfer dissociation (ETD) analysis was performed in the linear trap. Our results identify the modification sites and provide information on the sugar composition of the glycans as well as on the site specific oligosaccharide heterogeneity. We applied a novel iterative approach that allowed us to interpret the ETD dataset directly without making prior assumptions about the nature and distribution of oligosaccharides present in our glycopeptide mixture. Over 2500 unique N- and O-linked glycopeptides were identified from 453 proteins. The extent of microheterogeneity varied extensively, and up to 50 different oligosaccharide moieties were detected at a given Asn residue. Mucin-type O-glycosylation information was obtained for more than 100 proteins. We also detected EGF-domain-specific fucosylation, Tyr glycosylation and discovered a novel O-mannosylated protein: the transmembrane phosphatase Ptprz1.

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(235) "Hyposialylation in glomerulopathies is mitigated by N-acetylmannosamine therapy"

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Biallelic mutations in murine Gne, coding for UDP-GlcNAc 2- epimerase/ ManNAc kinase, the key enzyme in sialic acid biosynthesis, result in glomerular disease with podocyte effacement due to hyposialylation. We showed that oral supplementation with the sialic acid precursor N- acetylmannosamine (ManNAc) ameliorated the proteinuria and improved the podocyte foot process architecture and glomerular sialylation status of mutant mice. A panel of fluorescent- labeled lectins (including WGA, SNA, HPA, and PNA) applied to kidney sections, indicated aberrant sialylation of predominantly O- linked glomerular glycans in mutant mice kidneys; this normalized after ManNAc treatment.

Since hyposialylation has sporadically been suggested in human glomerulopathies, we applied the lectin panel to renal tissue sections from 40 patients with unexplained glomerulopathies. An unexpectedly high number of biopsies (8) had glomerular hyposialylation similar to that seen in our mouse model, indicating that this condition may occur relatively frequently, and also that ManNAc may be a therapy.

To gather more preclinical data, we induced podocyte hyposialylation in mice by intraperitoneal injection of (Vibrio cholera) sialidase, removing sialic acids from glycans. Sialidase- injected mice developed proteinuria and renal failure in a dose- dependent manner. Their glomerular glycoproteins were hyposialylated and their podocytes were effaced, similar to our Gne knock-in mouse model. Importantly, oral prophylaxis and treatment with ManNAc significantly reduced their proteinuria and podocyte injury.

Although the exact mechanisms and consequences of glomerular hyposialylation requires further study, oral ManNAc therapy could benefit patients with glomerular hyposialylation; ManNAc has minimal toxicity, is easily (orally) administered and could replace or supplement existing therapies. Moreover, ManNAc is currently being tested in a Phase 1 clinical trial for the treatment of the rare hyposialylation disorder GNE myopathy; it could be repurposed for trials in patients with glomerular hyposialylation.

(236) "Sugar nucleotide metabolic pathways in the blood stages of the malaria parasite"

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Carbohydrate structures play important roles in many biological processes, including cell adhesion, cell- cell communication, and host- pathogen interactions. Sugar nucleotides are activated forms of sugars used by the cell as donors for most glycosylation reactions. Although it is accepted that Plasmodium falciparum is unable to produce complex glycoconjugates other than glycosylphosphatidylinositol (GPI) anchors, the biosynthetic pathways for the biosynthesis of sugar nucleotides not related to the GPI structures are conserved in the parasite's genome. Their conservation strongly suggests that their products are involved in the biosynthesis of glycans not yet characterized. Using a liquid chromatography- tandem mass spectrometry- based method, we identified and quantified the pools of UDP- glucose, UDP- galactose, UDP- N- acetylglucosamine, GDP- mannose, and GDP-fucose in P. falciparum intraerythrocytic life stages. Assembling these data with the in silico functional reconstruction of the parasite metabolic pathways obtained from the P. falciparum annotated genome, we

have exposed new active biosynthetic routes crucial for further glycosylation reactions. Interestingly the genes encoding specific enzymatic steps cannot be identified in the parasite's genome, such as the GNAT activity that converts GlcN6P to GlcNAc6P and the source (and biological significance) of UDP- Gal. Fucose is a sugar present in glycoconjugates often associated with recognition and adhesion events. Thus, the GDP- fucose precursor is essential in a wide variety of organisms. P. falciparum presents homologues of GDP- mannose 4,6- dehydratase (GMD) and GDP- L- fucose synthase (FS) enzymes that are active in vitro, indicating that GDP- fucose is mostly formed by a de novo pathway that involves the bioconversion of GDP- mannose. Several proteins essential for the parasite motility and invasion and important vaccine candidates in clinical or preclinical development contain thrombospondin type-1 repeat TSR domains. These domains mediate protein interaction with their binding partners and in other organisms are acceptors of PoFUT2 O- fucosylation. The conservation and expression of an O- fucosyltransferase 2 (PoFUT2) homolog in the parasite's genome and the incorporation of tritiated GDP- fucose by the parasite in cell- free assays are strong arguments that suggest the presence of an uncharacterized mechanism of O-fucosylation active in P. falciparum. Furthermore, our preliminary data indicate that blood- stage parasites unable to synthesize GDP- fucose present a growth disadvantage in vitro.

(237) "Trials and tribulations of high throughput intact glycopeptide analysis"

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Glycosylation represents a rather unique form of post- translational modification (PTM). The modifying group may be a single sugar moiety, a relatively short oligosaccharide, or a large and complicated carbohydrate structure. Glycans featuring the same saccharide unit compositions may represent a wide variety of positional and linkage isomers. In addition, glycopeptides usually display a bewildering array of glycoforms that can vary quite widely even in glycan size. The analysis of glycopeptides is further complicated by the fact that the regularly used MS/ MS technique of collisional activation yields mostly carbohydrate fragments, and thus, cannot be used for peptide identification, while electron- transfer dissociation (ETD) yields sufficient information about the amino acid sequence but very little about the glycan.

We analyzed large intact glycopeptide datasets from several sources using Byonic, a newly available proteomics search engine, followed by manual analysis and interpretation. Byonic identifies glycopeptide spectra one at a time, using databases of protein sequences and glycan compositions; whereas, human experts can correlate and correct identifications over the entire data set and apply out- of- spectrum information. We also compared Byonic one- pass search results with Byonic and Protein Prospector multi- pass search results obtained by an iterative approach. The iterative approach first identifies the most prevalent glycan structures by performing very wide modification searches on extracellular proteins in the mixture, and then searches the spectra over again with a more focused list of glycan masses. Based on this work, we offer solutions for the semi- automatic interpretation of glycopeptide ETD spectra. We will also present examples of the difficulties one faces in glycopeptide analysis, and will outline some future directions.

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(238) "Structure basis for the catalytic mechanism of D-glucuronyl C5 epimerase"

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Heparan sulfate (HS) is a glycosaminoglycan which interacts with diverse signal molecules to exhibit essential physiological functions. L-Iduronic acid (IdoA) is an important protein-binding epitope in HS. D-glucuronic acid epimerase (GLCE) is a critical enzyme involving in HS synthesis, which converts D-glucuronic acid (GlcA) to IdoA. Here we report the homodimeric crystal structure of GLCE from zebrafish and structure of GLCE in complex with heparin hexasaccharide. Binding of the heparin hexasaccharide in the active site results in significant conformation change, including a slight shift of a ß-sheet rich domain, turning over of Q171-Q175 loop region and rotation of Y149, R154, D155 and R156 residues at the binding site. Based on the structural mutant analysis we demonstrate that each GLCE molecule has one catalytic domain which is a tyrosine and arginine rich cleft encircled by eight α-helixes. Y468, Y528, R531 and Y546 are located at active site and all play an important role in the epimeration reaction. In addition, we propose a precise mechanism of sugar epimeration by a proton abstraction and readdition method. This work provides novel evidence to help understanding the mechanism of HS synthesis and aids the development of therapeutical heparin mimics as well as the inhibitor by targeting GLCE.

(239) "The glycosylation status of the Pneumococcal serine-rich repeat protein modulates its contribution to adhesion, biofilm formation, and virulence"

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PsrP is a member of the Gram-positive bacteria Serine-rich repeat protein (SRRP) family, an inter- and intra-species adhesin that binds to lung cells and mediates biofilm formation in vivo. PsrP is expressed as part of the pathogenicity island psrP-secY2A2 that also

encodes 10 glycosyltransferases (GTFs: glyABCDEFG, nss, and gtf-AB) and 7 components of a non-conical SecY2A2 transport system. These genes are required for the glycosylation of PsrP and its transport to the bacterial surface. Importantly, the identity of the glycoconjugates on PsrP and how glycosylation impacts PsrP function was unknown. We first determined that PsrP is most highly produced during biofilm growth and predominantly carries N-acetylglucosamine along with traces of galactose; these might be part of a complex oligosaccharide. We next examined how glycosylation impacts PsrP stability by Western blot using antibody and biotinylated lectins, adhesion to A549 lung cells, in vitrobiofilm formation, and virulence in a mouse model using isogenic GTF mutants. ĐgtfA- and ĐgtfB- had undetectable PsrP and behaved in all experiments identical to the complete locus mutant, ĐpsrP-secY2A2-, suggesting an unstable protein. Đnss- had detectable PsrP but was attenuated in all aspects tested. ĐglyC-and ĐglyF- had undetectable PsrP, but remained virulent. Deletion of glyA- had no impact on any virulence property assayed. ĐglyE- was only attenuated for biofilm formation, whereas ĐglyB- was attenuated for adhesion and biofilm formation in vitro, but remained virulent. Finally, glycosylation of PsrP in ĐglyD- was not evident using our methods, yet PsrP was detectable with antibody and functional. We subsequently attributed these diverse phenotypes to altered presentation of PsrP on the bacterial cell surface. But also suggest that there is a yet uncharacterized virulence property for PsrP. These studies indicate PsrP function is strongly modulated by its glycosylation status. PsrP most likely plays distinct roles in S. pneumoniae that carry distinct GTF combinations.

(240) "Fucosylated and charged glycan epitopes of the malaria vector Anopheles gambiae"

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The mosquito Anopheles gambiae is the most efficient vector of human malaria. Two fundamental steps are required for the Plasmodium parasite development and transmission. Both steps, the binding of the parasite in the insect vector and in the vertebrate host cells, have been connected to the presence of oligosaccharide structures (glycans). By applying a homology search, we found three putative fucosyltransferases in the A. gambiae genome. All three fucosyltransferases, which are potentially involved in glycan biosynthesis, were cloned and expressed in Pichia pastoris GS115 cells. The recombinant and purified fucosyltransferases have been used in MALDI-TOF MS based assays to determine various enzyme properties (pH and temperature optima as well as ion dependence). The characterization of all three fucosyltransferases showed that in comparison to many other glycosyltransferases, the A. gambiae fucosyltransferases have no absolute requirement for any special divalent cation cofactor. By the combination of several methods including Western and Lectin Blot analysis as well as immunofluorescence staining of A. gambiae sections and dissected tissues, we observed the presence of in vivo fucosyltransferase products. In addition, N- and O-glycan analysis of mosquito larvae confirmed the occurrence of fucosylated glycan epitopes and revealed novel charged glycan epitopes in A. gambiaeas well as in another mosquito species Aedes aegypti. These experimental data on the biosynthesis as well as the hitherto unique charged modifications of mosquito glycans are a valuable basis for further investigations of molecular host-parasite interactions.

(241) "Identification of novel biomarkers via three different glycan-related microarrays"

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Glycocalyx, literally meaning 'sugar coat', is an extracellular polymeric coating surrounding many prokaryotic and eukaryotic cells consisting of glycoproteins, glycolipids, proteoglycans and glycosaminoglycans. The constituents of the glycocalix play an important role in the process of cell signaling, virus transfection, and immunity. In the latter case, the glycocalix can both form a physical barrier to infection can help immune cells to distinguish between the body's own healthy cells and transplanted tissues, diseased cells, or invading organisms. Just as antibodies recognize specific epitopes, proteins known as 'lectins' recognize specific glycans (i.e., each lectin binds only specific sequences of carbohydrate units in the polysaccharide or oligosaccharide chain), as free glycans and/or glycans covalently attached to target glycoproteins as a post-translational modification. Because of the relatively low affinity between glycans with lectins and the complexity of glycosaminoglycans, there are only a few assay kits available to probe the function of the glycocalyx or its constituents and to facilitate glycoprotein biomarker development. Here we introduce three distinct glycan-related microarrays to help identify glycan and/or glycoprotein biomarkers in patient serum/plasma and other sample types:

1. Antibody Lectin Glycosylation (ALG) Array: This array combines the antigen specificity of antibody arrays with the glycan-specific binding of bio-lectins to screen for glycoproteins in patient serum or other sample types. More than 1,000 antibodies were printed on glass slides array in duplicate. The specificity of the capture antibodies determines the identity of the bound glycoprotein. However, by using more than forty bio-lectins, the presence and degree of glycosylation on target protein biomarkers can be determined, including the identification of distinct glycosaminoglycan sugar moieties that are attached.
2. Lectin Array: More than 40 important lectins were printed in two concentrations (duplicate spots for each concentration) on glass slides. The lectin array surface was incubated with samples containing glycoproteins and/or glycans that were either direct-labeled with fluorescent dyes or with biotin-

labeled samples, which were subsequently detected using streptavidin-conjugated fluorescence dyes. The specificity between the lectin and glycan/ glycoproteins can be verified by specific inhibitors, and MS/MS can be used to determine the identities of novel glycoprotein biomarkers that exhibit interesting signals detected by the Lectin array.

3. Glycan Array: More than fifty important glycans were printed in two concentrations (duplicate spots for each concentration) on glass slides The proteins/ biomarkers which can bind to the unique glycan sequences from the samples could be identified by using biotinylated samples or biotinylated antigen-specific antibodies with a streptavidin-conjugated fluorescent dye. The specificity of interactions between the glycans and bound proteins can be verified by inhibitors, and MS/MS can be used to determine the novel lectins that exhibit interesting signals detected by the Glycan Array.

(242) "Analytical characterization of exopolysaccharides produced by novel bacteria from the genera Acidovorax and Luteibacter"

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Endosymbiont bacteria are omnipresent in nature, yet they are still little explored and an underestimated source of novel microorganisms. The acquired knowledge about the gut microbiota can be used as controlling agent for pathogen vectors or industrially important crop plagues. Microbial communities are almost always associated with biofilm forming bacteria, and the characterization of the exopolysaccharides, which comprises most of the biofilm, might give an advantage for understanding and control of those communities. Furthermore, the discovery of new polysaccharide based products might find excellent applications in a wide range of economically relevant areas. In this context, this work aimed to identify the strains with most vigorous growth and EPS producing abilities among the successfully isolated strains from the gut of Diabrotica speciosa, a polyphagous insect that attacks a large number of important crops in South America, and to elucidate the EPS structure produced by them. Two strains were selected and identified by 16s rRNA gene sequences, namely 2B which was identified as Acidovorax sp. with 91% similarity, and 2E which was identified as Luteibacter sp. with 93% similarity, both of which have not their EPS characterization described in literature so far. 1H NMR analysis has shown that EPS from Luteibacter has a major constituent, with anomeric proton shift at 5.37ppm. An interesting observation is the characteristic signals for O-acetylated substituents. 3JH1-H2 of 4 Hz and GC-MS data from per-O-acetylated derivatives indicates that the major constituent is α-D-Glcp. Further analysis of coupling constants from the other anomeric proton shifts indicate the existence of monosaccharides with both α and β pyranose configurations. For the EPS from Acidovorax sp., 1H and 13C NMR show a complex heteropolysaccharide structure, with at least 6 different signals for the anomeric proton and carbon spectrum region. Comparing with GC-MS chromatograms of sugar standards, rhamnose, arabinose, xylose and glucose have been identified. Also unknown alditol acetates were observed, indicating the presence of more monosaccharides on the structure. Further studies by 1D and 2D NMR, PMAA and (S)-(+)-2-Butanol derivatives by GC-MS and MALDI-TOF/TOF are being performed to complete elucidation.

(243) "A novel baculovirus vector for the production of non-fucosylated recombinant glycoproteins in insect cells"

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The ability to glycosylate recombinant proteins is an important attribute of baculovirus- insect cell expression systems, but some insect cell lines produce recombinant glycoproteins with core α 1,3- fucosylated N- glycans, which are highly immunogenic and render products unsuitable for human use. To address this problem, we exploited the function of a bacterial enzyme, GDP-4-dehydro-6- deoxy-D- mannose reductase (Rmd), which consumes the precursor to GDP-L- fucose. We expected this enzyme to indirectly block glycoprotein fucosylation by blocking the production of GDP-L- fucose, which is required as the donor substrate for this process. Initially, we engineered two different insect cell lines to constitutively express Rmd and isolated subclones with fucosylation- negative phenotypes. Surprisingly, however, we also found that the fucosylation- negative phenotypes induced by Rmd expression were unstable in insect cell lines, indicating that a host cell engineering approach would not be successful. Thus, we ultimately constructed a novel baculovirus vector designed to express Rmd immediately after infection and to facilitate downstream engineering for expression of any glycoprotein of interest later in infection. We used this vector to produce a daughter encoding anti- CD20- IgG and found, in contrast to an Rmd- negative control, that insect cells infected with this virus produced a non-fucosylated form of this antibody. These results indicate that the problem of immunogenic core α 1,3- fucosylated N- glycan production associated with the baculovirus-insect cell system can be solved using the Rmd baculoviral vector produced in this study. In conjunction with existing glycoengineered insect cell lines, this vector extends the utility of the baculovirus- insect cell system to include therapeutic glycoprotein production. Finally, this new vector extends the utility of the baculovirus-insect cell system to include production of recombinant antibodies with enhanced effector functions, due to its ability to block core α 1,6- fucosylation.

(244) "Sialylation of Thomsen-Friedenreich antigen is a noninvasive blood-based biomarker for GNE myopathy"

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GNE myopathy is an adult- onset progressive myopathy resulting from mutations in GNE, encoding the key enzyme of sialic acid biosynthesis. The pathomechanism of GNE myopathy likely involves aberrant sialylation, since administration of sialic acid itself or its precursor, N- acetylmannosamine (ManNAc), rescued the hyposialylation of GNE myopathy mice. Recently, clinical trials for GNE myopathy patients were initiated. A robust, noninvasive biomarker is highly desirable for diagnosis of GNE myopathy and for evaluating response to therapy. Since GNE myopathy muscle biopsies demonstrated hyposialylation of predominantly O- linked glycans, we analyzed the O- linked glycome of patients' plasma proteins using mass spectrometry. Most patients showed increased plasma levels of the O- linked Thomsen- Friedenreich (T)- antigen and/ or decreased amounts of its sialylated form, ST- antigen. Moreover, every patient we analyzed had an increased ratio of T- antigen to ST- antigen when compared to unaffected individuals. Importantly, the T/ ST ratios were normalized in GNE myopathy patients treated with intravenous immunoglobulin (which are highly sialylated proteins) or with self- administered ManNAc (off- label use), indicating response to therapy. Natural history and clinical trial data will reveal whether T/ ST ratios correlate with clinical outcomes. These findings not only highlight plasma T/ ST ratios as a robust blood- based biomarker for GNE myopathy, but also help explain the pathology and adult onset of the disease. Specifically, a shortage of total sialic acid occurs in GNE myopathy, likely later in life. Some glycans (N- linked) may be preferably sialylated over others (O- linked). Proteins with significant O-linked glycosylation will predominantly be affected and contribute to the phenotype, many aspects of which remain to be identified.

(245) "Analysis of Circulatory Glycosyltransferases: Divergence Between Human and Mouse"

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Glycosyltransferases, usually residing within the intracellular secretory apparatus, circulate in the blood. The association of these circulating enzymes with pathological states, including malignancies and inflammatory conditions prompts further understanding of the functional roles of these enzymes in biologic processes, such as the potential for dynamic modification of glycans on distal cell surfaces and in extracellular compartments. Moreover, circulating glycosyltransferases have not been systematically studied among different species. Characterization of the species-specific relative expression of these blood-borne enzymes in different mammals is useful to understand their roles in fundamental biologic processes.

Here, we describe a systematic evaluation of blood-borne sialyl-, galactosyl- and fucosyltransferase activities that act upon the four common terminal glycan precursor motifs, GlcNAc monomer, Gal(ß3)GlcNAc, Gal(ß4)GlcNAc, and Gal(ß3)GalNAc, to produce more complex glycan structures. Data from radioisotope assays and detailed product analysis by sequential tandem mass spectrometry show that blood has the capacity to generate many of the well-recognized and important glycan motifs, including the Lewis, sialyl-Lewis, H-, and Sialyl-T antigens. While many of these glycosyltransferases are freely circulating in the plasma, human and mouse platelets are important carriers for others, including ST3Gal-1 and ß4GalT. Human platelets are also carriers for large amounts of ST6Gal-1 and the α3-sialyl to Gal(ß4)GlcNAc sialyltransferases, both of which are conspicuously absent in mouse platelets. Identification of key convergence and divergence of blood-borne glycan-synthetic capabilities between humans and mice offers novel perspectives on the capacity to modify glycans in the extracellular milieu.

(246) "Glucuronyl glycans are essential for α-Klotho-dependent Na+/K+-ATPase recruitment and FGF signaling"

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α- Klotho (α- Kl) regulates mineral homeostasis bybinding to the Na +/ K +- adenosine triphosphatase (Na +/ K +- ATPase) complex,fibroblast growth factor- receptor 1 (FGF- R1), and FGF-23.In this study, we investigated how α- Kl binds to such a variety of distinct partnersand found that α- Kl functions as a novel glucuronide- binding lectin.We discovered that a portion of the Na +/ K +- ATPase ß- subunit and FGF- R1 are glucuronylatedon their N- glycans and that FGF-23 is modified with a novel glucuronyl O- glycan. α- Kl uniquely recognizes these glycans for specific and physiological binding.The glucuronyl glycan directly binds α- Kl and drives it to form stable protein- protein interactions. α- Kl thereby recruits Na +/ K +- ATPase complexes from trans- Golgi networks to the cell surface,and accumulates circulating FGF-23 to activate FGF- R1.

(247) "Bacterial nonulosonates for glyco-engineering purposes: Efficient enzymatic synthesis of N-acetimidoyl and N-acetimidoyl-N-methyl nonulosonate sugars"

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In addition to presenting sialic acids on their surface, bacteria can incorporate sialic acid-like sugars (5,7-diacetamido-3,5,7,9-tetradeoxy-nonulosonate derivatives) into their virulence-associated cell-surface glycoconjugates, such as lipopolysaccharide

(LPS), capsular polysaccharide, pili and flagella. For example, the sialic acid-like sugars 5-N-acetimidoyl-7-N-acetyl-legionaminic acid and 5-N-acetimidoyl-5-N-methyl-7-N-acetyl-legionaminic acid are found within the LPS O-chains of the Gram-negative bacteria Legionella pneumophila, causative agent of Legionnaire`s disease, and the flagellins of Campylobacter jejuni, a Gram-negative gastrointestinal bacterial pathogen. In addition to legionaminic acid forms, C. jejuni also possesses pseudaminic acid sugars within its flagellins, such as 5-N-acetyl-7-N-acetimidoyl-pseudaminic acid. In this work, we have purified and biochemically characterized 3 candidate biosynthetic enzymes from C. jejuni 11168 enabling efficient synthesis of the amidine and N-methyl containing nonulosonates described above. Specifically, two stereo-selective amidotransferases and one S-adenosyl methionine dependent methyltransferase were characterized, in which all 3 were found to utilize CMP-sugar substrates. Importantly, these findings provide a facile method for efficient large-scale synthesis of bacterial sialic acid mimics, such as the zwitterionic amidine-containing nonulosonates, which may now be practically utilized within various biomedical and glyco-engineering applications.

(248) "Dual functionality of O-GlcNAc transferase is required for Drosophila development"

Xiaowei Zheng; *University of Dundee*

Post- translational modification of intracellular proteins with N- acetyl glucosamine (O- GlcNAc) has been implicated in diverse cellular functions, and is catalysed by O- GlcNAc transferase (OGT). ogt has been shown to be required for normal development in a range of organisms. Drosophila OGT is encoded by super sex combs (sxc), mutants of which produce pupal lethality, although it is not clear whether this is through loss of cellular O- GlcNAc or loss of the OGT protein. Here we have dissected the catalytic versus non- catalytic roles of OGT in Drosophila development. We show that OGT catalytic activity is required for development up to late pupal stages, while further development requires as yet uncharacterised non- catalytic functions of OGT.

(249) "A Novel Metabolic Pathway For Mannose-6-P 2nd Messengers"

Mark Lehrman; UT Southwestern Medical Center at Dallas

Free mannose-6-phosphate (M6P) can be a 2nd-messenger signaling molecule (JBC 280:17901; MBoC 22:2994), responding to endoplasmic reticulum (ER) stress caused by chemicals or viral infection, and requiring the stress kinase IRE1 to elevate M6P. M6P signals trigger hydrolysis of the LLO "G3M9Gn2-P-P-Dolichol", ostensibly depriving infectious virus of LLO for glycosylation of envelope proteins. M6P 2nd messengers require glycogenolysis, using glycogen phosphorylase (GP) to convert glycogen to Glc-1-P (G1P). This presents a confounding problem!! How can glycogenolysis for M6P signaling avoid cross-circuiting with glycogenolysis via classical cAMP/protein kinase A signaling, which generates Glc-6-P (G6P)/Fru-6-P (F6P) for glycolysis? Both processes require GP, and the conventional pathway from glycogen to M6P also involves G6P and F6P intermediates.

We propose an alternative, distinct metabolic path from glycogen to M6P 2nd messengers, with three important features. (i) Phosphomannose isomerase (PMI) normally converts F6P to M6P. Unlike the conventional path, PMI is not required for M6P 2nd messengers. (ii) In the conventional path, phosphoglucose isomerase (PGI) is upstream of PMI and converts G6P to F6P. Unlike PMI, M6P 2nd messengers do require PGI. (iii) Sedoheptulose-7-P (S7P) is normally restricted to the pentose phosphate pathway, where it can be metabolized into two equivalents of F6P. S7P is also increased by ER stress in a manner requiring GP, and its consumption involves PGI. PGI has also been reported to possess some ability to convert F6P to M6P, and we speculate that IRE1 may regulate PGI for this purpose. Together, generation of M6P 2ndmessengers (A) appears distinct from M6P synthesized via conventional cAMP signaling (B):

A. ER stress-IRE1-GP-glycogenolysis-G1P-?-S7P-F6P-PGI*-M6P (no PMI)
B. cAMP-PKA-GP-glycogenolysis-G1P-G6P-PGI-F6P-PMI-M6P (no S7P)

Thus, the proposed pathway is a "mash-up" of conventional glycogen metabolism and the pentose-phosphate pathway. We still cannot explain how GP/glycogenolysis for ER stress signaling can be distinguished from the same activities for cAMP signaling; how G1P can yield S7P; the nature of PGI*; or how F6P in the IRE1 pathway is diverted from glycolysis. However, the model does provide a framework for avoiding metabolic cross-circuiting of the two signaling pathways, which respond to fully independent environmental cues. NIH-GM038545.

(250) "Bacterial glycosylation in the deep-sea: Functional characterisation of N-linking oligosaccharyl transferases encoded by deep-sea vent bacteria"

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Fifteen years ago, N- linked protein glycosylation was believed to be restricted to eukaryotes and archaea. However, the discovery and functional characterisation of the Campylobacter jejuni N- glycosylation machinery around the turn of the millennium demonstrated that such post- translational modifications also occur in bacteria. Since then, additional systems encoded by other Campylobacter species as well as by species of Helicobacter and Desulfovibrio have been identified and characterised. Intriguingly, recent genome sequencing of bacteria from deep- sea vent habitats identified species encoding orthologues of the C. jejuni oligosaccharyl transferase (OTase) enzyme PglB. Here, we present evidence that pglB orthologues from two deep- sea vent epsilon- proteobacteria and one deep- sea vent delta- proteobacterium encode functional OTase enzymes. As these bacteria require specialised growth conditions, we employed a recombinant approach and analysed the enzymes

by expression in E. coli together with a lipid- linked oligosaccharide substrate and an acceptor protein. We found that analogous to the C. jejuni PglB, the epsilon-proteobacterial OTase enzymes possess relaxed specificity regarding the glycan substrate, and require the extended, 5 amino acid "bacterial" N- glycosylation sequon "D/ E- Y- N- X- S/ T" for activity. Interestingly, these enzymes displayed more stringent requirements regarding the acceptor protein than the C. jejuni PglB, and were only able to glycosylate a subset of acceptor proteins and sequons tested. As this could be attributed to an experimental bias stemming from the use of C. jejuni glycoproteins as acceptors, we also undertook an initial analysis of the putative glycoproteome of the two deep- sea vent epsilon- proteobacteria, and identified more than 100 putative N- glycoproteins.

This data expands the world of bacterial N- linked glycosylation systems, and may in turn lead to the discovery and development of novel tools for use in bacterial glycoengineering. It also raises interesting questions regarding the evolution of bacterial protein N- glycosylation and the role of these modifications in the biology of the bacteria.

(251) "Detecting O-GlcNAc using in vitro sulfation"

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O- GlcNAc glycosylation, the covalent attachment of N- acetylglucosamine to serine and threonine residues of proteins, is a post- translational modification that shares many features with protein phosphorylation. O-GlcNAc is essential for cell survival and plays important roles in many biological processes (e.g., transcription, translation, cell division) and human diseases (e.g., diabetes, Alzheimer's disease, cancer). However, detection of O- GlcNAc is challenging. Here, a method for O- GlcNAc detection using in vitro sulfation with two GlcNAc- specific sulfotransferases, CHST2 and CHST4, and the radioisotope 35S is described. Sulfation on free GlcNAc is first demonstrated, and then on O- GlcNAc residues of peptides as well as nuclear and cytoplasmic proteins. It is also demonstrated that the sulfation on O- GlcNAc is sensitive to OGT and OGA treatment. The labeled samples are separated on SDS- PAGE and visualized by autoradiography. Overall, the method is sensitive, specific and convenient.

(252) "Production of novel pneumococcal glycoconjugate vaccines using a bacterial oligosaccharyltransferase"

Laura E. Yates, Emily J. Kay , Sherif Abouelhadid , Dominic C. Mills , Adrian J. Jervis, Jon Cuccui, Brendan W. Wren; *LSHTM*

Streptococcus pneumoniae causes serious respiratory disease, bacteraemia and meningitis, and it has been estimated that globally one million children under the age of five die of pneumococcal disease each year. Existing conjugate vaccines are efficacious, but protect against only a limited number of the 93 known serotypes of S. pneumoniae. Furthermore, the most prevalent serotypes vary according to location, meaning a single vaccine composition is unlikely to be optimal for all countries, and high production costs are liable to hinder availability in developing countries where the incidence is highest. The identification of a bacterial oligosaccharyltransferase in Campylobacter jejuni (CjPglB) and the subsequent functional transfer of this enzyme into Escherichia coli led to the possibility of utilizing this recombinant system for the production of novel glycoconjugates. We have termed this process protein glycan coupling technology (PGCT).

The capsular polysaccharide coding locus from serotype 4 S. pneumoniae was cloned and recombinantly expressed in E. coli, resulting in production of a polymerised polysaccharide that could be detected using a serotype- specific antiserum. The oligosaccharyltransferase CjPglB was found to be capable of transferring this recombinant polysaccharide to an established acceptor protein from C. jejuni, confirming that PGCT can be used for the production of unique pneumococcal glycoconjugates. Furthermore, the flexibility of this system means any protein may potentially be incorporated into a glycoconjugate. Taking advantage of the extensive research that has been conducted into immunogenic S. pneumoniae proteins as vaccine candidates, a non- toxic derivative of the pore- forming toxin pneumolysin was modified for use with the PGCT system. When expressed in combination with the serotype 4 polysaccharide and the oligosaccharyltransferase, this protein was also glycosylated. The range of proteins and capsular polysaccharides tested is being expanded, and the resulting novel glycoconjugates are being tested as vaccine candidates. It is hoped PGCT will form a powerful new tool for the production of novel pneumococcal glycoconjugate vaccines.

(253) "Crystallization of a Mimivirus Collagen Glucosyltransferase"

Kelvin B. Luther, Thierry Hennet; *University of Zürich*

We recently reported the characterization of a collagen hydroxylysine glucosyltransferase from the giant virus Mimivirus. This collagen glucosyltransferase activity defines a novel form of core glycosylation in collagen. The glucosyltransferase activity is associated with a distinct domain in a bi- functional enzyme possessing collagen lysyl- hydroxylase activity in a second domain. Mimivirus and human collagen sequences can be modified by both the lysyl- hydroxylase and glucosyltransferase activities. We now report the crystallization of the Mimivirus collagen lysyl-hydroxylase/ glucosyltransferase enzyme expressed in E. coli and purified by affinity and size exclusion chromatography. Initial crystallization conditions from nano- drop experiments are being scaled up and optimized in preparation for structural characterization of the enzyme.

(254) "Development of a Sample Preparation Method for Quantification of alpha-Galactose Content in Therapeutic Fc-fusion Proteins."

Oliver Pabonan, Tomasz Baginski; *Genentech*

Fc-fusion proteins are molecules comprised of the Fc domain of an antibody fused to another type of

protein, such as cytokines or receptor extracellular domains. Fc-fusion proteins often exhibit multiple N-linked glycosylation sites, which result in significantly more heterogeneous glycosylation profiles than those typically observed from monoclonal antibodies.

Certain CHO-derived Fc-fusion proteins have been reported to display the alpha-Gal epitopes (galactose-a-1,3-galactose) at the terminal ends of N-glycans. The presence of alpha-Gal epitopes may lead to immunogenic response and hypersensitivity reactions in patients. Therefore, it is important to monitor for the potential presence of alpha-Gal epitopes during the development of new Fc-fusion proteins.

This poster describes the optimization of a sample preparation method employing a-galactosidase digestion for release of the alpha-galactose from the therapeutic Fc-fusion protein followed by its subsequent separation and quantification by HPAEC-PAD. The developed method was applied during the selection of clones for a new CHO-derived Fc-fusion protein, currently in early stage of clinical development. The levels of alpha-Gal in some commercially available products were also determined and compared to the material obtained from the screened clones.

(255) "A workflow for analyzing site-specific glycan heterogeneity"

Matthew Renfrow[1], Audra Laube[1], Jennifer Cushing[1], Stacy Hall[1], Blake Moore[1], Monica Stinnett[1], Zina Moldoveanu[1], Scott Peterman[2], Qing Wei[1], Amol Prakash[2], Jiri Mestecky[1], Bruce Julian[1], Milan Raska[3], Jan Novak[1]

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Defining site- specific glycan heterogeneity is still recognized as a challenging analytical task that reflects the difficulty of reliably identifying all glycooforms present in a given glycoprotein isolation. This task is all the more complicated in proteins that have several or even multiple sites of glycosylation. While release of glycans from the protein provides glycan heterogeneity information, the context of the protein and in many cases the relevant biological information is lost. We have developed a workflow for the analysis of site-specific protein glycosylation heterogeneity for heavily N- and O- glycosylated proteins that takes advantage of a variety of high resolution and tandem mass spectrometry techniques to 1) identify multiple sites of modification within a single protein isolation, 2) define the glycan heterogeneity at specific sites, and 3) allow comparison across multiple samples to identify differences in site- specific glycan heterogeneity. We will demonstrate this workflow on the analysis of the heavily O-glycosylated IgA1 isolated from the serum of healthy controls and patients with IgA nephropathy, a chronic kidney disease that involves aberrantly O-glycosylated IgA1 in the pathogenesis. Profiling multiple IgA1 O-glycoforms (including isomers) across several fractions of a single patient sample, patient sample sets, and different patient cohorts is a difficult informatics process that we have tackled with the assistance of a modified Pinpoint software package (ThermoFisher Scientific). The result is a series of IgA1 O- glycopeptide panels that show distinct ranges, relative distributions, and site- specific localizations between IgAN patients and healthy controls. We will also demonstrate the application of our workflow on the analysis of the heavily N- glycosylated HIV-1 env gp120 protein (25 sites of N-

glycosylation and 1 site of O- glycosylation). By developing a workflow to quickly identify and characterize these sites of glycosylation, our goal is to find glycan differences and commonalities between different cell lines as well as early transmitted versus chronic stage forms of the virus. Our current results will demonstrate our ability to reliably provide quantitative comparison of individual sites of glycosylation across a range of samples for both N- and O- type glycosylation.

(256) "Analysis of gp120 Glycosylation Heterogeneity"

Audra H. Laube[1], Qing Wei[1], Stacy Hall[1], Katerina Zachova[2], Zhi-Qiang Huang[1], Lydie Czernekova[2], Zina Moldoveanu[1], Milan Raska[2], Scott Peterman[3], Amol Prakash[3], Jan Novak[1], Matthew B. Renfrow[1]

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HIV-1 gp120 is part of the envelope glycoprotein on the HIV capsid. It plays a key role in virus entry into host cells and as a shield against neutralizing antibodies. gp120 is difficult to characterize based on the number of N- glycans (25 sites) and heterogeneity of glycans that can be present on a specific site at any given time. We have previously demonstrated that expression of gp120 in a variety of cell lines results in differential N- glycosylation of the many potential sites within the protein that affects envelope recognition by polyclonal antibodies from the sera of HIV-1- infected subjects[1]. Individual sites of N- glycosylation within gp120 can be identified by N- glycan consensus sequence motifs and established methods for the localization of sites of glycosylation by use of ETD, HCD, and CID fragmentation methods. Still, the multiple sites of glycosylation within gp120 make assessing the individual site heterogeneity a large informatics task. Further complicating this analysis is the high rate of mutation that alters the amino acid sequence as well at the N-glycan heterogeneity of gp120 as the virus evades immune response. We have developed an informatics workflow to quickly identify these sites of glycosylation. Our goal is to find N- glycan differences and commonalities between different cell lines as well as early transmitted versus chronic stage forms of the virus. In our current analysis, we have successfully identified all possible sites by LC- MS/ MS methods. The range of N- glycans at Individual sites are elucidated by inspection of the MS1 by use of a modified Pinpoint software to identify low abundance glycoforms and allows the creation of targeted gp120 N- glycopeptide panels that can be readily compared across multiple samples. This work will report on the successful implementation of our workflow to identify sites of N- glycan variability between various preparations of HIV-1 env gp120.

[1] Raska, el al, (2010) JBC, 285:20860-9.

(257) "Comprehensive study of Proteome and Glycoproteome changes in Platelet upon Activation and Aspirin Treatment"

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Platelets are fragments of megakaryocytescells and circulate in the blood. Platelets aggregate and form a thrombus during vascular injury. Platelet glycoproteins play a major role in in platelet activation, aggregation and secretion. In Cardiovascular diseases, thrombus formation may leads to ischemic events such as myocardial infarction or stroke. Aspirin has anti- platelet activity and is used in low doses by patients with high risk of cardiovascular diseases. Aspirin irreversibly inhibits the enzyme COX by protein acetylation resultingin reduced platelet activity. A subset of population has high platelet activity of aggregation after aspirin treatment. This population is at a high risk factor for the recurrence of ischemic events.

To investigate the effects of aspirin on platelet and its secretion, Platelet proteome and N- glycoproteome were analyzed. Platelets and its secretion were analyzed with and without stimulation through collagen in the presence and absence of aspirin. We employed quantitative LC- MS/ MS based global proteomics and glycoproteomics approaches for the quantitative comparative analysis of platelet and platelet secretome. The study method incorporated isobaric tags for relative and absolute quantitation (iTRAQ), solid- phase enrichment of glycopeptides, two- dimensional liquid chromatography, nanoelectrospray ionization and high resolution tandem mass spectrometry using Easy Spray Q Exactive followed by data analysis using Proteome Discoverer. Results were analyzed using 1% False Discovery Rate (FDR).

In our preliminary studies, we were able to identify over 5000 global proteins and 350 glycoproteins from platelet. After Platelet activation, a significant decrease in platelet secretion of certain protein was observed in the presence of the aspirin upon activation with collagen. We also identified decrease in secretion of certain novel glycoprotein upon aspirin treatment; these glycoproteins were not identified in global proteomics study. In order to confirm that this effect was largely independent of the donor, the experiment was repeated with 5 different donors. Very similar platelet releasate profiles were observed for all donors.Globalproteomics and glycoproteomics study of the platelets and their secretion could provide valuable clues for better understanding the underlying mechanisms of proteins and their glycosylation upon platelet activity and impact on aspirin.

(258) "Analysis of O-linked oligosaccharides of high-molecular mass proteins from cyst fluid of ovarian cancer"

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Most of ovarian cancer cases are diagnosed in very late stages when mortality rate is high. To reduce the mortality from ovarian cancer, new biomarkers are needed, that can improve the detection of ovarian cancer in earlier stages. Cyst fluids are potentially a very good source for identification of new biomarkers, since it would be enriched for proteins secreted by the cancer tissue.

We have previously analyzed the O-glycome of ascites from patients with ovarian cancer. Now we are following this up by describing the O-glycome cyst fluid samples from different types (benign, borderline and malignant) and stages (stage I-IV). The O-linked oligosaccharides released from acidic glycoproteins from cyst fluids were analyzed by negative-ion graphitized carbon Liquid Chromatography (LC) coupled to Electrospray tandem Mass spectrometry (ESI-MSn). It was found that O-linked oligosaccharide from cyst fluid resemble O-linked oligosaccharides found in ascites with its high level of sulphation and sialylation. We also identified blood groups antigens in the O-linked glycome, and we are currently investigating their relation to the subtypes and severity of the cancer.

This study and comparison of O-linked glycosylation of mucins type molecules from cyst fluids can lead to discovery of new biomarkers for better detection and treatment of ovarian cancer. The presence of blood group antigens and its relation to cancer indicates that there is a need for personalized diagnostic as well as personalized treatment in the future health care system, an area where glycobiology will have a high impact.

(259) Altered Glycosylated Synaptomatrix Signatures and Synaptic Architecture in a Drosophila Classic Galactosemia Disease Model

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Classic galactosemia (CG) is an autosomal recessive disorder that results from loss of galactose-1-phosphate uridyltransferase (GALT), the Leloir pathway enzyme which catalyzes conversion of galactose 1-phosphate + UDP-glucose to glucose 1-phosphate + UDP-galactose. UDP-galactose 4'-epimerase interconverts UDP-galactose to UDP-glucose, and is responsible for the biosynthesis of UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. All four UDP-sugars are essential donors for the synthesis of glycoproteins and glycolipids that heavily decorate cell surfaces and the extracellular space. In addition to acute, potentially lethal neonatal symptoms in CG, mature patients develop substantial motor and cognitive impairments. Previous studies suggest an association between neurological phenotypes and glycosylation defects, with CG described as Congenital Disorder of Glycosylation (CDG) with a combined defect in the assembly and processing of N-glycans. Based on this prediction, our goal has been to test for impacts on behavioral traits, synaptic development and glycosylated synaptomatrix formation using a GALT-

deficient Drosophila CG model. We tested larval coordinated movement, adult locomotion and daily activity levels, and found that loss of dGALT impairs the larval rollover response, adult locomotion and adult activity levels. We characterized larval neuromuscular junction (NMJ) structure, and found that GALT-deficient larvae exhibit structural overelaboration with increased bouton and branch number, increased process length and decreased bouton diameter and inter-bouton distance. Dietary galactose supplementation as well as co-removal of the dGALK gene establish environmental and genetic modifiers of these behavioral and neurological phenotypes. We assayed the NMJ extracellular environment (synaptomatrix) with a panel of lectin labeling and found profound alterations in the glycan composition in the absence of dGALT, including significant reductions in the amount of galactosyl and N-acetyl galactosamine residues, and fucosylated HRP epitopes. Taken together, these results reveal synaptic glycosylation defects as a consequence of UDP-gal deficiency in CG, and synaptic architecture defects in the GALT-deficiency condition, and raise the intriguing possibility that inhibition of GALK activity might benefit the CG disease state. Similar mechanistic defects during synaptogenesis may account for the neurological pathogenesis characterizing a wide array of CDGs.

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(260) Modeling Protein Glycosylation and Associated Diseases

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The Golgi plays a key role in glycosylation of proteins. Enzymes vital to glycosylation are distributed non-uniformly throughout the Golgi. This distribution is maintained by retrograde vesicle transport. Mutations in the proteins responsible for this transport result in altered glycomes and consequently human disorders classed as congenital disorders of glycosylation.

Glycan profiles exhibit a large degree of heterogeneity, primarily due to the lack of a template, and the resulting rather complex biosynthetic machinery. To understand how this complexity is managed during biosynthesis, a modelling approach is warranted. The model uses an L-system that is a mathematical formalisation often used for complex repeating biological structures. Rewriting is central to L-systems, where complex objects are generated by successively replacing parts of a starting object using a set of rules. The starting object in our model is the GlcNAc2Man9 glycan, and the rules describe enzymatic removals and additions of sugars in the Golgi. For the model to replicate biological data, 'prior parameter distributions' contain information on enzyme location, amount and rate gathered from microarray and literature data. This initial model had limited success replicating the experimentally measured glycan profile. Systematic manipulation of the enzyme parameters using a Markov chain Monte Carlo method improved the fit between the model and glycan profiles. The obtained post-fitting enzyme parameters (the 'posterior' enzyme distribution and rates) can inform us on the true layout of the biosynthetic machinery.

To prevent model bias towards a single glycome, perturbations of the glycosylation machinery were also modelled. These included the inhibition of Ð-mannosidase II using swainsonine, and knock-down of the Cog4 subunit of the conserved oligomeric Golgi complex that coordinates enzyme distribution within the Golgi. Creating iterations of the model using a variety of glycomes will result in a predictive tool that can be used to calculate a glycome for a given mutation or inhibitor without the need for biological samples. A further use of the modeling will be to describe perturbations of the biosynthetic machinery that are experimentally difficult to determine.

Keywords: glycosylation; computational modeling